

INFLUENCE OF COLD  
ON HOST-PARASITE INTERACTIONS

PART II



Editor

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# EFFECT OF LOW AMBIENT TEMPERATURES ON SPECIFIC AND NONSPECIFIC RESISTANCE

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## ABSTRACT

Adult albino mice (Mus musculus) have been employed in a series of experiments to determine the effect of acute and chronic low temperature exposure on resistance to bacterial disease. Disease organisms used were Klebsiella pneumoniae and Staphylococcus aureus. Animals challenged with these organisms had been previously immunized with the specific agent or had been pretreated with zymosan or Escherichia coli endotoxin. Groups of mice kept at 21° C were compared to similarly treated groups kept at 2° C for 30-45 days. Also, mice challenged at 21° C were then placed at 2° C. The mice challenged at 2° C were kept at this same temperature, caged either singly or in groups. The results show that under these conditions specific immunization affords significant protection as compared to nonspecific immunization of the animals. The degree of resistance induced by nonspecific immunization is significant by comparison with the control animals. The extent of protection is decreased if the animals are acutely cold stressed as compared to chronic cold stress. Also, specific immunization does not protect animals as well if they are caged individually at the cold temperature as compared to being caged in groups; thus, psychological factors of isolation should not be disregarded. It is concluded that specific immunization affords increased resistance compared to nonspecific immunization; however, the degree is dependent on factors such as grouping and chronicity of exposure.

Reported results vary concerning the effect of exposure of experimental animals to cold on virus-induced diseases. In some cases resistance has been found to decrease, in other cases resistance has been found to remain unchanged or to increase. Different routes of challenge, viral agents, animal species employed, and variations in caging, such as individual or grouped, are among the variables which may account for apparently diverse results reported. Experiments were conducted with one strain of adult albino Mus musculus to determine the effect of acute and chronic exposure of these animals on induced viral disease. The mice were kept at either 2° C or room temperature (ca. 21° C). The viral agent chosen was a strain of Coxsackie B-5 which will infect the mice following intraperitoneal injection. Results indicate that: (1) Acute exposure followed by challenge results in lowered resistance. (2) Specific immunization affords significantly increased protection which is not reduced by acute exposure. (3) Nonspecific immunization enhances resistance above that shown by untreated animals, but the extent of resistance is less than that achieved by specific immunization. If the animals are first acclimatized to cold and then challenged, results are changed in the following manner: (1) Acclimatized mice are capable of withstanding challenge doses that

## MIYA, MARCUS AND PHELPS

kill challenged and acutely exposed animals. (2) Specific immunization increases resistance of mice kept at 2° C, and this immunity is of the same magnitude as in animals kept and immunized at room temperature. (3) Nonspecific immunization does not increase resistance of cold acclimatized mice.

Numerous reports on the influence of environmental temperature on host-parasite relationship have appeared in the following literature: Pasteur, Joubert, and Chamberland, 1878; Lillie et al., 1937; Fay and Henny, 1938; Armstrong, 1938 and 1942; Smith and Fay, 1939; Bischoff and Long, 1939; Sarracino and Soule, 1942; Fuller, Brown, and Mills, 1941; Goldfeder, 1941; Wallace, Wallace, and Mills, 1942 and 1944; Mills and Schmidt, 1942; Muschenheim et al., 1943; Sulkin, 1945; Ipsen, 1952; Walker and Boring, 1958; Sulkin et al., 1960; Miya et al., 1962; Miraglia and Berry, 1962; Previte and Berry, 1962. There is general agreement that the physical environment can alter or influence the course of disease; however, isolating the specific factors involved remains difficult. Consequently, the reports of results obtained by workers in this field are frequently conflicting even though the same challenge agents and animal species may have been employed.

During the past two years experiments have been conducted in our laboratory to test the hypothesis that preceding or following exposure to low temperatures, the capacity of experimental animals to resist local or systemic infectious diseases is (a) unchanged or (b) increased or decreased. Presumably, changes observed would be mediated by humoral, cellular, or vaguely defined physiological factors which may be isolated or otherwise identified for study.

Our experiments have proceeded along three lines: (1) investigation of the effect of low ambient temperature on host-parasite relations in both unacclimatized and acclimatized animals following experimental infection; (2) investigation of alteration of specific and nonspecific resistance of animals exposed to cold stress; (3) investigation of the influence of low ambient temperature on the progress of viral neoplastic disease in mice.

## SPECIFIC AND NONSPECIFIC RESISTANCE

### MATERIALS AND METHODS

In all the experiments adult albino mice (Mus musculus) were randomized as to sex, age, and weight. The average weight of the animals at the initiation of any experiment was 21 gm. The ambient temperatures employed were 21° C and 2° C. The temperatures of the rooms did not vary more than  $\pm 1.5^{\circ}$  C. The mice were placed at 2° C following treatment and challenge at 21° C (acute exposure of unacclimatized animals) or exposed to 2° C for varying periods, treated, challenged, and observed at 2° C (acclimatized animals).

Klebsiella pneumoniae was obtained from departmental stock cultures, and Staphylococcus aureus, strain Fritchie, was obtained from Doctor R. D. Higginbotham, University of Texas Medical Branch, Galveston, Texas. The organisms were maintained on blood agar. Single colonies were transferred to tryptose phosphate broth (Difco) or solid media and incubated at 37° C for 18 hours when needed.

Viruses used in these experiments were obtained from departmental stock and from Doctor Duard L. Walker, University of Wisconsin, Madison, Wisconsin. The viruses were propagated and assayed on monkey kidney cells using technic described by others (Dulbecco and Vogt, 1954; Youngner, 1954; Bubel, 1958; Bailey, 1960).

Vaccines were prepared for specific immunization from the bacterial and viral agents by the use of formalin inactivation. The mice were immunized with intraperitoneal injections of the vaccines contained in 0.1 ml volume. Seven days were allowed to lapse before challenge. Zymosan (lot OB 298, Fleischmann Laboratories, Stamford, Connecticut) and Escherchia coli endotoxin (Difco 0293, E. coli 055:B5) were used as nonspecific immunizing agents. The details of the preparation and immunization schedules have been previously described (Miya, Marcus, and Perkins, 1961; Marcus et al., 1961a; Marcus et al., 1962).

Measurements of the mouse core, shell, and upper respiratory cavity temperatures were obtained with calibrated probes (Electric



Universal Thermometer, type TE3, Chemical and Pharmaceutical Industry Company, Inc., New York). The rectal probe was inserted 2 cm (Marcus et al., 1961b).

Oxygen uptake studies were conducted by employing the Warburg constant volume respirometer technique (Umbreit, Burris, and Stauffer, 1957). The substrate was 2.5 ml of tryptose phosphate broth. Standardized amounts of bacteria were added in a volume of 0.5 ml. The center well contained 0.2 ml of 20 per cent KOH plus a 1 cm<sup>2</sup> fluted filter paper. Flasks were set up in duplicate.

## RESULTS

### Effect of Varying Temperatures

In order to assess the effects of acute and chronic low temperature stress on mechanisms of specific and nonspecific resistance to microorganisms, it became apparent that information concerning the behavior of the challenge agents at low temperatures was necessary. It has been observed that certain small animals undergo a considerable drop in core temperature when placed in an environment in which the ambient temperature is considerably less than the normal core temperature. Since specific information concerning the metabolic and growth behavior of the challenge agents (K. pneumoniae and S. aureus) at temperatures lower than 37° C was lacking, it became apparent that data were necessary for the interpretation of experiments involving specific and nonspecific resistance mechanisms at core temperatures less than normal.

Growth of the bacteria was determined by employing turbidity as a function of time. Turbidity of the growing cultures was measured in a Klett-Summerson photoelectric colorimeter with a blue filter. The numbers of organisms present for any given turbidity measurement was determined from a standard curve based on the assumption that each organism present divides at the same time; the generation time for these experiments is defined

# SPECIFIC AND NONSPECIFIC RESISTANCE

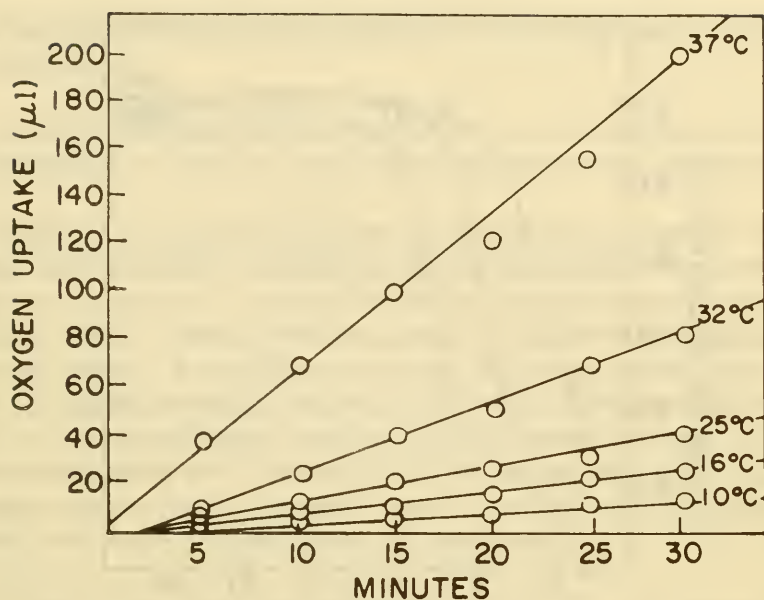


Figure 1. Effect of varying temperatures on oxygen uptake of *Klebsiella pneumoniae*.

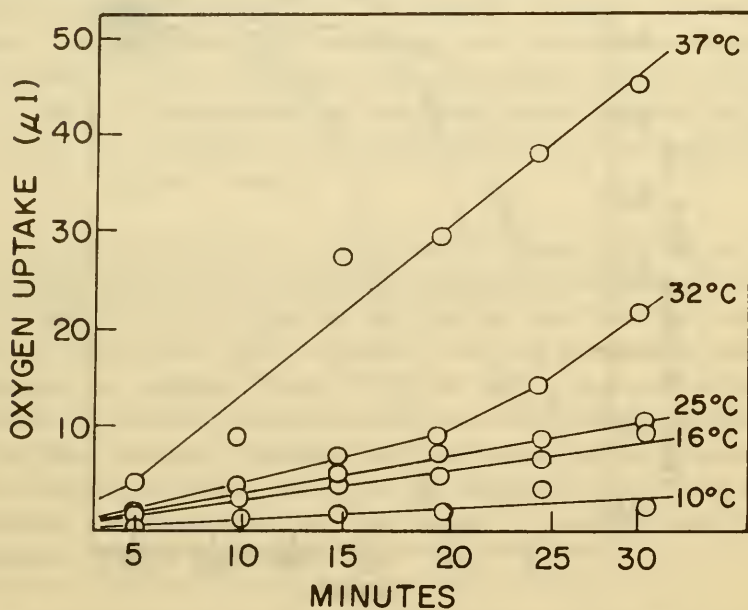


Figure 2. Effect of varying temperatures on oxygen uptake of *Staphylococcus pyogenes aureus*.

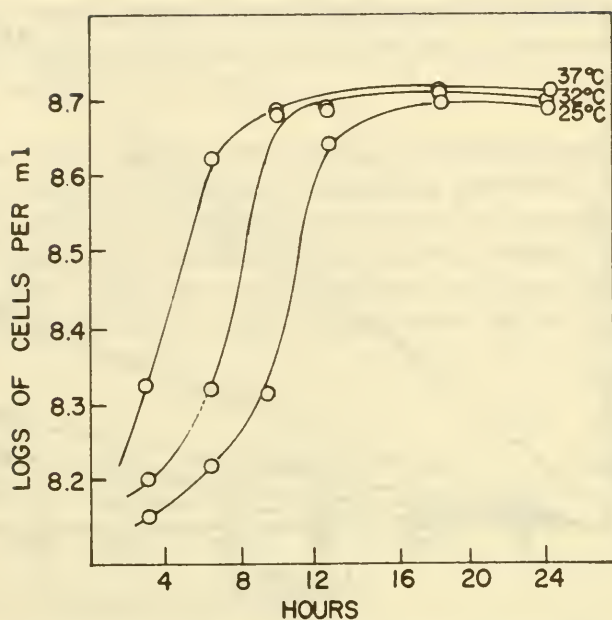


Figure 3. Effect of varying temperatures on growth of *Klebsiella pneumoniae*.

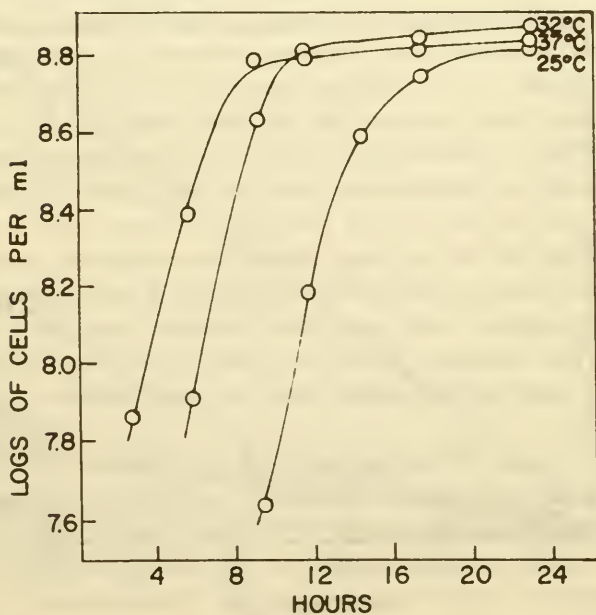


Figure 4. Effect of varying temperatures on growth of *Staphylococcus pyogenes aureus*.



## SPECIFIC AND NONSPECIFIC RESISTANCE

as the time required for the numbers of bacteria present to double in population.

Constant temperature water baths which maintained the specified temperature  $\pm 0.5^{\circ}$  C were employed in all the experiments.

Effect of varying temperatures on oxygen uptake. Figures 1 and 2 show that a number of different curves are obtained when oxygen uptake is plotted as a function of time for a given temperature. The results appear identical with the strains employed in K. pneumoniae and S. aureus. A significant decrease in oxygen uptake occurs at  $32^{\circ}$  C compared to that occurring at  $37^{\circ}$  C. At temperatures below  $32^{\circ}$  C, the oxygen uptake rates are again lessened, but little change is noted between  $20^{\circ}$  C and  $25^{\circ}$  C. It is of interest that the S. aureus oxygen uptake is significantly lower than that of K. pneumoniae when the same temperatures are compared.

Effect of varying temperatures on growth and generation times. Calibrated cuvettes (13 x 100 mm) containing 5 ml of tryptose phosphate broth were seeded with a standard loop (0.01 ml) of broth inoculum obtained from an 18-hour culture. The optical density was measured at zero time and thereafter at various increments of time. Duplicate sets of cuvettes were employed. The number of organisms for any given period of time was determined by reference to a standard curve. The results are shown in Figures 3 and 4.

It is seen that similar results were obtained with the strains of K. pneumoniae and S. aureus. In both cases, families of curves were obtained. The slopes of the curves are not significantly different from each other, indicating that the generation time at temperatures ranging from  $37^{\circ}$  C to  $25^{\circ}$  C are not markedly affected by different temperatures, but a noticeable increase in the lag phase is seen. No measurable increase in turbidity occurred at temperatures below  $25^{\circ}$  C. It is possible that, for the time employed, multiplication did not occur or was too slow to be measured by this technique.

Studies on Klebsiella Pneumoniae Passed Through Mice Maintained at Low Ambient Temperatures

The previous section revealed information concerning the metabolic behavior of the bacterial challenge agents at different temperatures; however, it was not possible to predict the metabolic behavior and virulence of the organisms when given to an animal that was acclimatized to the cold. In order to see if any changes did occur, the following experiment was carried out.

A single isolated colony of K. pneumoniae was inoculated into tryptose phosphate broth (Difco) and incubated for 18 hours at 37° C. One tenth ml of broth suspension of organisms per mouse was given intraperitoneally. At the end of 24 hours, the surviving mice were sacrificed by decapitation. The peritoneal cavities were opened aseptically and the peritoneal exudate was removed. The exudate was reinjected into a number of mice after a sample had been seeded to heart infusion blood agar plates (Difco) for isolation and identification procedures. This procedure was repeated each day for 7 days. The inoculated mice were kept at either 21° C or 2° C during this time of organism passage. The mice had been kept at these respective temperatures for 40 days prior to injection with the organisms. The K. pneumoniae strain isolated from the animals kept at 2° C was incubated at room temperature (21° C) while the organisms isolated from the animals kept at 21° C were incubated at 37° C.

The K. pneumoniae passed through mice maintained at 2° C was compared with the K. pneumoniae passed through mice maintained at 21° C with respect to their metabolic behavior and virulence. It is seen in Figure 5 that incubation of both strains at 37° C results in similar growth behavior curves when comparing organisms isolated from the animals maintained at 2° C or at 21° C. Slightly different growth curves in terms of comparison of the two strains were obtained when these organisms were incubated at 32° C (Figure 6). The K. pneumoniae isolated from animals maintained at 2° C showed growth curves almost identical with those obtained at 37° C incubation, whereas the organisms isolated from mice maintained at 21° C show a definite decrease in growth maximum in addition to an increased lag phase. These results suggested that

# SPECIFIC AND NONSPECIFIC RESISTANCE

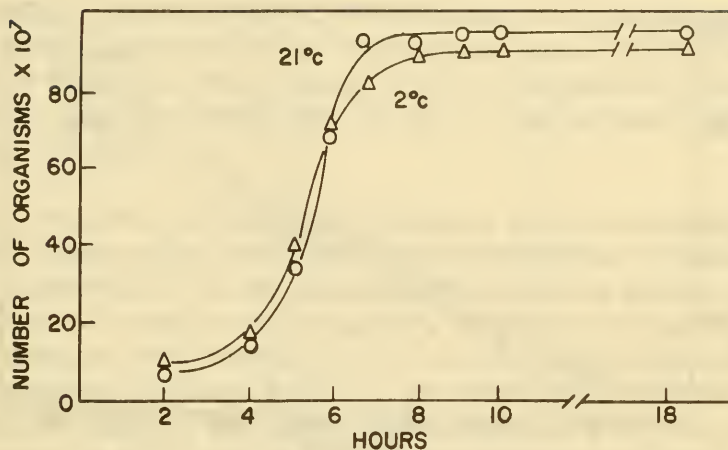


Figure 5. Effect of 37° C incubation temperature on growth behavior of K. pneumoniae isolated from mice maintained at 2° C and 21° C.

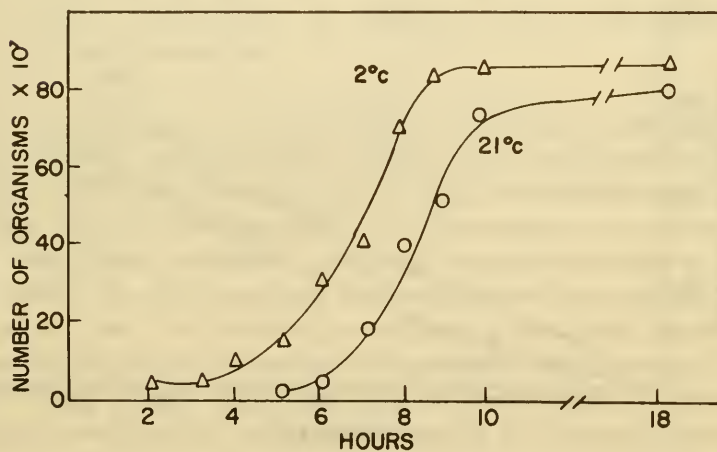


Figure 6. Effect of 32° C incubation temperature on growth behavior of K. pneumoniae isolated from mice maintained at 2° C and 21° C.

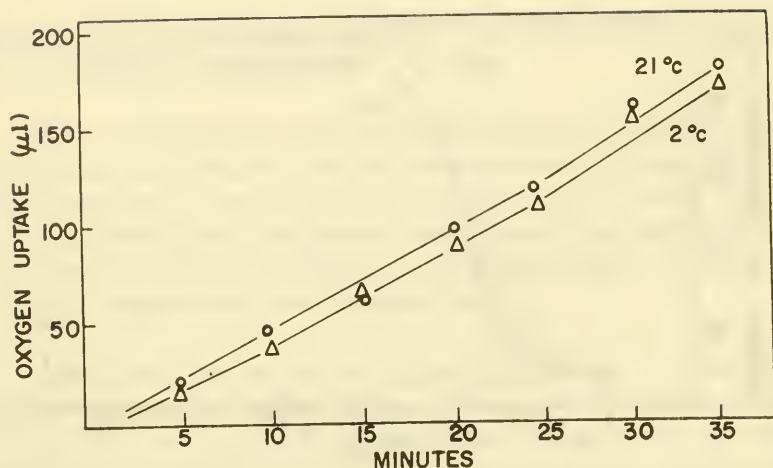


Figure 7. Effect of 37° C incubation temperature on oxygen uptake of K. pneumoniae isolated from mice maintained at 2° C and 21° C.

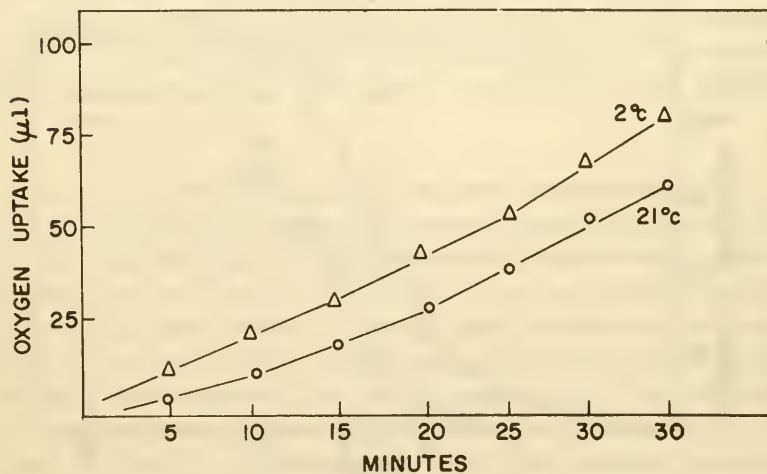


Figure 8. Effect of 32° C incubation temperature on oxygen uptake of K. pneumoniae isolated from mice maintained at 2° C.



## SPECIFIC AND NONSPECIFIC RESISTANCE

an organism isolated from a low temperature acclimatized animal can grow equally well at the optimal temperature of  $37^{\circ}\text{C}$  or at reduced incubation temperatures of  $32^{\circ}\text{C}$ , whereas the organism isolated from an animal kept at  $21^{\circ}\text{C}$  does not have this dual growth capacity.

The situation becomes more confusing when one looks at the oxygen uptake curves. When both the isolated strains of organisms are incubated at  $37^{\circ}\text{C}$ , the oxygen uptake curves are similar qualitatively and quantitatively (Figure 7). When both isolated strains of organisms were incubated at  $32^{\circ}\text{C}$ , the oxygen uptake of the K. pneumoniae isolated from the low temperature acclimatized animals was greater than that of the organisms isolated from mice maintained at  $21^{\circ}\text{C}$ . This is what one would expect since the growth curves exhibit these same changes; however, note that quantitatively, the amount of oxygen uptake for both isolates is approximately one-half when incubation is carried out at  $32^{\circ}\text{C}$  compared to  $37^{\circ}\text{C}$ . This implies that the isolate from the mice kept at  $2^{\circ}\text{C}$  is more efficient metabolically since it can grow as rapidly at  $32^{\circ}\text{C}$  as it can at  $37^{\circ}\text{C}$ , yet requires only one-half the amount of oxygen to do so (Fig. 8).

The  $\text{LD}_{50}$  values of both isolates are summarized in Table I. No significant differences or changes in the virulence of the organisms were observed.

Mouse temperature measurements. In order to determine the effect huddling had on mouse temperature measurements, the following experiment was conducted. Mice were placed at  $2^{\circ}\text{C}$  or  $21^{\circ}\text{C}$  in cages containing either a single mouse or a group of five mice. Core, skin, and upper respiratory cavity temperatures were taken hourly for the first four to five hours on the first day, then once a day for 8 to 14 days, and finally on the forty-fifth day of exposure. Figure 9 shows that the presence of five mice in one cage at  $2^{\circ}\text{C}$  results in temperature measurements that increase gradually, reaching an initial maximum in 2 to 4 hours after exposure. Generally, the rectal temperature is consistently higher than the upper respiratory cavity, but it does not appear to be significantly greater. The skin temperatures are considerably less than the rectal or upper respiratory cavity temperatures, so the



MIYA, MARCUS AND PHELPS

| Temperature of Experiment | Dose | Organism | Mortality Ratio | LD <sub>50</sub> (95% Confidence Limits) |
|---------------------------|------|----------|-----------------|--|
| 2° C                      | 1040 | P        | 10/10           | 20<br>(14.1 - 28.4)                      |
|                           | 104  |          | 9/10            |  |
|                           | 10.4 |          | 4/10            |  |
|                           | 1.04 |          | 0/10            |  |
| 2° C                      | 1040 | NP       | 10/10           | 35<br>(14.6 - 84.0)                      |
|                           | 104  |          | 8/10            |  |
|                           | 10.4 |          | 3/10            |  |
|                           | 1.04 |          | 0/10            |  |
| 21° C                     | 1040 | P        | 10/10           | 35<br>(15.9 - 77.0)                      |
|                           | 104  |          | 7/10            |  |
|                           | 10.4 |          | 4/10            |  |
|                           | 1.04 |          | 0/10            |  |
| 21° C                     | 1040 | NP       | 10/10           | 35<br>( 3.5 - 350)                       |
|                           | 104  |          | 4/10            |  |
|                           | 10.4 |          | 5/10            |  |
|                           | 1.04 |          | 0/10            |  |

Table I. LD<sub>50</sub> values of *Klebsiella pneumoniae* isolated from mice maintained at 2° C(P) and 21° C(NP) following intraperitoneal injection.

three temperature curves appear to parallel each other.

In Figure 10 are presented the results of temperature measurements obtained on singly-caged mice maintained at 2° C. Again the three temperature curves parallel each other, and the temperature difference is of the following descending order: rectal, upper respiratory cavity, skin. The rectal temperatures are not significantly greater than the upper respiratory cavity temperatures, but they do show consistently higher values. Similar to the results obtained with the grouped mice, the skin temperatures of the singly-caged mice are considerably lower than the rectal and upper respiratory cavity temperatures. It is of interest to note that an initial rise in temperature occurs within one hour after exposure to the low ambient environment, and is then followed by a sharp drop in temperatures which reaches a maximal fall by four hours post-exposure. From this point the temperatures gradually rise to reach stability by 24 hours post-exposure. The time of stabilization appears to be the same as that required for the grouped mice.

# SPECIFIC AND NONSPECIFIC RESISTANCE

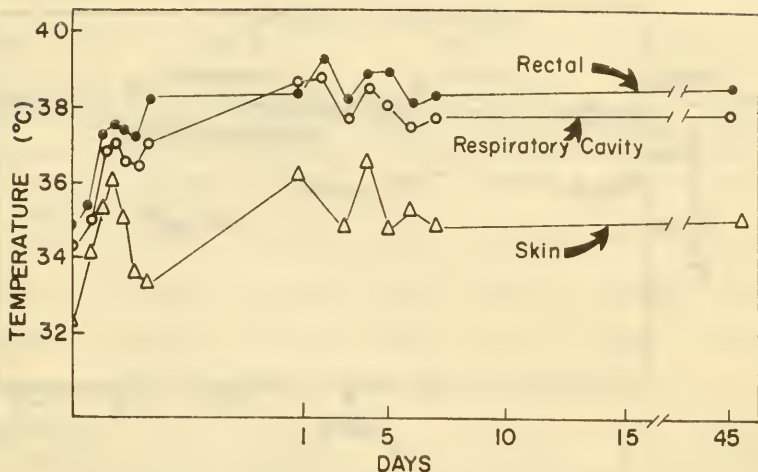


Figure 9. Effect of grouping (5 mice/group) on average temperatures of mice maintained at  $2^{\circ}\text{C} \pm 1^{\circ}\text{C}$ .

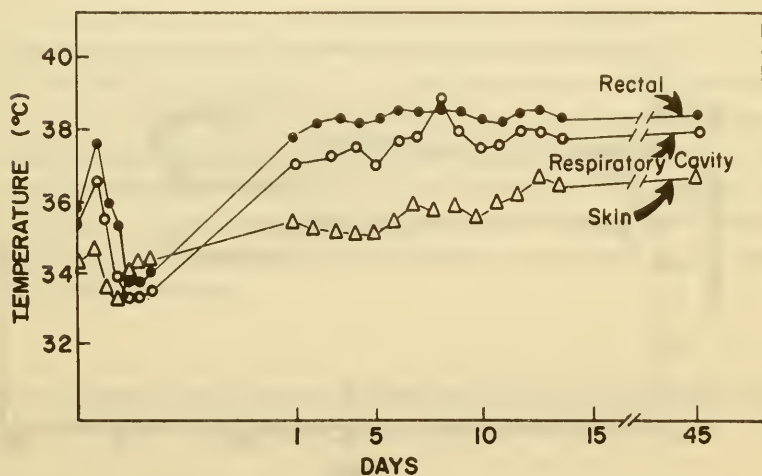


Figure 10. Effect of single caging on average temperatures of mice maintained at  $2^{\circ}\text{C} \pm 1^{\circ}\text{C}$  (average of 10 mice).

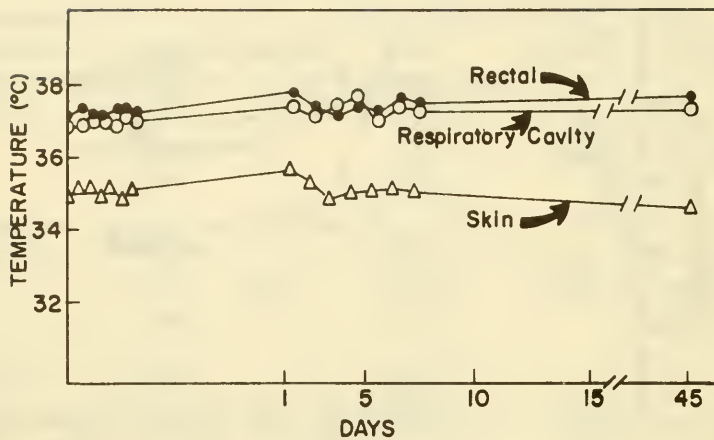


Figure 11. Effect of grouping (5 mice/group) on average temperatures of mice maintained at  $21^{\circ}\text{C} \pm 1^{\circ}\text{C}$ .

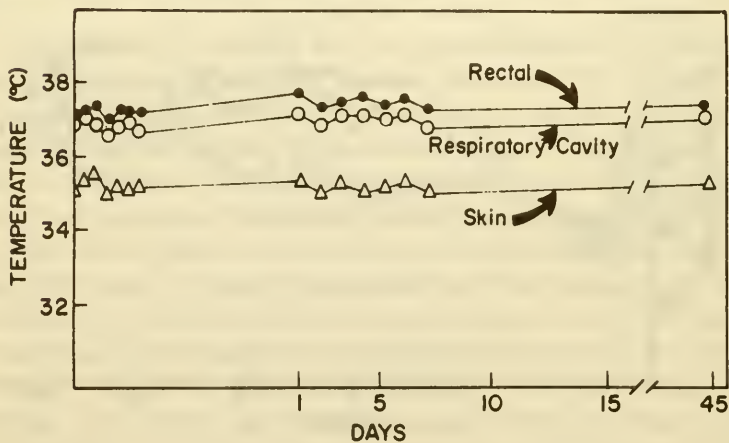


Figure 12. Effect of single caging on average temperatures of mice maintained at  $21^{\circ}\text{C} \pm 1^{\circ}\text{C}$  (average of 10 mice).

## SPECIFIC AND NONSPECIFIC RESISTANCE

| Number of<br>Organisms | Ambient Temperatures |                |                   |                |                  |                |
|------------------------|----------------------|----------------|-------------------|----------------|------------------|----------------|
|                        | 21° C<br>(grouped)   |                | 2° C<br>(grouped) |                | 2° C<br>(single) |                |
|                        | normal               | immu-<br>nized | normal            | immu-<br>nized | normal           | immu-<br>nized |
| 4.3                    | 8/10*                | 0/10           | 7/8               | 0/10           | 8/8              | 6/10           |
| 43                     | 9/10                 | 0/10           | 9/10              | 0/10           | 9/10             | 7/10           |
| 430                    | 10/10                | 0/10           | 10/10             | 0/8            | 10/10            | 6/10           |
| 4300                   | 10/10                | 0/10           | 10/10             | 1/8            | 10/10            | 6/10           |
| Agglutinin<br>titer**  | 0                    | 1:32           | 0                 | 1:32           | 0                | 1:32           |

Table II. Effect of acute cold stress (2° C) on mortality of mice challenged with *Klebsiella pneumoniae*. Unacclimatized animals injected at room temperature, then placed at noted temperatures. \*7 day mortality (dead/total). \*\*Titer before challenge.

When temperatures are measured on mice grouped five per cage and maintained at 21° C, the results shown in Figure 11 are obtained. The rectal and upper respiratory cavity temperatures are about the same in magnitude, while the skin temperatures range about 2° C to 2.5° C less. The temperature curves again parallel each other, but they do not exhibit any marked fluctuations as seen with mice maintained singly or grouped at 2° C. Essentially, the temperatures of the mice remained quite constant throughout the experiment. The data charted in Figure 12 demonstrate that mice singly caged and kept at 21° C exhibited temperature curves that were almost identical with those obtained with grouped mice maintained at the same ambient temperature.

### Effect of Acute and Chronic Exposure to Low Temperatures on Survival of Mice Challenged with *Klebsiella Pneumoniae*

Acute exposure experiments. Mice were immunized at room temperature and then were challenged with varying numbers of *K. pneumoniae*. Immediately following this procedure, the mice were transferred to an ambient temperature of 2° C. The con-

## MIYA, MARCUS AND PHELPS

| Number of<br>Organisms                              | Ambient Temperatures |                |                     |   |                      |                |
|---|----------------------|----------------|---------------------|---|----------------------|----------------|
|   | 21° C                |                | 2° C                |   | 2° C                 |                |
|   | (grouped)<br>normal  | immu-<br>nized | (grouped)<br>normal | immu-<br>nized  | (single)<br>normal   | immu-<br>nized |
| 5.6   | 3/10*                | 0/10           | 10/10               | 0/10  | 7/9                  | 0/10           |
| 56  | 4/10                 | 0/10           | 9/9                 | 1/10  | 7/8                  | 0/10           |
| 560   | 10/10                | 0/10           | 10/10               | 2/10  | 6/6                  | 1/10           |
| 5600  | 10/10                | 0/10           | 10/10               | 2/10  | 9/9                  | 1/10           |
| LD <sub>50</sub><br>(95% Con-<br>fidence<br>limits) | 56<br>(52-59)        | -              | -                   | 51 x 10 <sup>4</sup><br>(49 x 10 <sup>4</sup> -<br>52 x 10 <sup>4</sup> ) | 4.1<br>(3.8-<br>4.4) |                |
| Agglutinin<br>titer**                               | 0                    | 1:32           | 0                   | 1:16  | 0                    | 1:16           |

Table III. Effect of chronic cold stress (2° C) on mortality of mice challenged with *Klebsiella pneumoniae*. Animals maintained for 45 days, and immunized at noted temperatures. \*7 day mortality (dead/total).

trol groups of mice were kept at 21° C. The results in Table II show that immunization protected the grouped animals. In contrast, the immunization procedure was less effective in mice singly-caged. The nonchallenged stress controls placed at 2° C did not die when grouped, but some (10 per cent) did when singly caged, suggesting that huddling of animals enables a more favorable outcome when animals are acutely stressed by low ambient temperatures.

Chronic exposure experiments. Two groups of mice were placed at 2° C and 21° C for periods of 45 days before being immunized. One week after the last immunizing injection, the mice were challenged with varying numbers of viable *K. pneumoniae*. The results in Table III show that mice were significantly protected by the immunization procedure. The titer of agglutinin antibody formed by mice chronically exposed to 2° C was comparable to that formed by animals kept at 21° C. It is interesting that no significant differences in



## SPECIFIC AND NONSPECIFIC RESISTANCE

mortality of grouped or singly-caged normal animals was observed. Immunization protected the counterpart groups to the same extent. It can be concluded from these results that the mice chronically exposed to an ambient temperature of 2° C were able to form agglutinin antibody and that the immunization procedure offered significant protection against the challenge organisms. In contrast, the normal mice chronically exposed to 2° C were adversely affected by K. pneumoniae; that is, smaller numbers of organisms caused increased mortality whether the animals were grouped or caged individually.

### Effect of Acute and Chronic Low Temperature Stress on Survival of Mice Challenged with Staphylococcus Aureus

Four strains of S. aureus were tested for their virulence for mice via the IP route in order to determine which would be the most suitable for subsequent studies. The Fritchie strain was found to be the most virulent. The LD<sub>50</sub> was  $25 \times 10^7$  organisms with 95 per cent confidence limits of  $13 \times 10^7$  to  $47 \times 10^7$ . Deaths of animals challenged with one LD<sub>50</sub> of the Fritchie strain usually occurred within 5 to 10 hours after challenge; however, the experiments were not terminated until 7 to 10 days had elapsed.

In the acute exposure experiments, mice were immunized at 21° C. Immediately following the IP challenge, the mice were kept either at 21° C or transferred to 2° C. The mice that were transferred to the low ambient temperature were caged either in groups of 10 animals or as individuals. The results of a typical experiment in Table IV show that a dose-response effect from S. aureus, Fritchie strain, is obtainable whether mice are exposed to the cold environment or kept at room temperature. Note that immunization is effective in protecting the challenged mice kept at 21° C and in groups at 2° C; in contrast, immunization did not afford protection to mice caged individually. Although not listed in the table, non-challenged cold stress control mice caged individually did not die during the experimental period (mortality ratio, 0/10).

Mice that were chronically exposed to low ambient temperatures

MIYA, MARCUS AND PHELPS

| Number of<br>Organisms      | Ambient Temperature                       |                |                   |                |  |  |
|-----------------------------|---|----------------|-------------------|----------------|--|--|
|                             | 21° C<br>(grouped)                        |                | 2° C<br>(grouped) |                | 2° C<br>(single)                           |  |
|                             | normal                                    | immu-<br>nized | normal            | immu-<br>nized | normal                                     | immu-<br>nized                             |
| $1.67 \times 10^9$          | 10/10*                                    | 2/10           | 10/10             | 1/10           | 10/10                                      | 8/10                                       |
| $1.67 \times 10^8$          | 6/10                                      | 0/10           | 10/10             | 0/10           | 10/10                                      | 7/10                                       |
| $1.67 \times 10^7$          | 0/10                                      | 0/10           | 0/10              | 0/10           | 4/10                                       | 1/10                                       |
| $1.67 \times 10^6$          | 0/10                                      | 0/10           | 0/10              | 0/10           | 0/10                                       | 0/10                                       |
| LD <sub>50</sub> and<br>95% | $10 \times 10^7$<br>( $4.9 \times 10^7$ ) | -              | -                 | -              | $3.5 \times 10^7$<br>( $1.2 \times 10^7$ ) | $6.5 \times 10^7$<br>( $1.7 \times 10^7$ ) |
| Confidence<br>Limits        | to<br>$22 \times 10^7$ )                  |                |                   |                | to<br>$9.8 \times 10^7$ )                  | to<br>$24 \times 10^7$ )                   |
| Agglutinin<br>titer         | 0   | 1:32           | 0                 | 1:32           | 0  | 1:32                                       |

Table IV. Effect of acute cold (2° C) on mortality of mice challenged with *Staphylococcus aureus*, strain Fritchie. Unacclimatized animals immunized and challenged at room temperature, then placed at noted temperatures. \*7 day mortality (dead/total).

either in groups or individually were kept at this temperature for 21 days. These acclimatized mice were immunized at this temperature and were subsequently challenged one week after the last immunizing injection. Table V illustrates that the immunization of the animals kept at 21° C afforded protection to the challenged animals. However, immunized mice kept in groups or individually at 2° C did not obtain the benefits of immunization as well as the animals kept at 21° C. In addition, the singly caged animals at 2° C showed even less benefit from immunization than did the grouped counterpart animals kept at the same low temperature. As with the *K. pneumoniae* experiments, agglutinin antibody formation was not impaired in mice chronically exposed to low ambient temperatures, whether the animals were caged in groups or individually. In contrast to the *K. pneumoniae*

# SPECIFIC AND NONSPECIFIC RESISTANCE

| Number of<br>Organisms                    | Ambient Temperatures |                |                         |                         |                          |                          |
|---|----------------------|----------------|-------------------------|-------------------------|--------------------------|--------------------------|
|   | 21° C<br>(grouped)   |                | 2° C<br>(grouped)       |                         | 2° C<br>(single)         |                          |
|   | normal               | immu-<br>nized | normal                  | immu-<br>nized          | normal                   | immu-<br>nized           |
| $1.64 \times 10^9$                        | 7/8*                 | 4/10           | 10/10                   | 6/10                    | 10/10                    | 10/10                    |
| $1.64 \times 10^8$                        | 7/8                  | 0/10           | 5/10                    | 4/10                    | 6/10                     | 7/10                     |
| $1.64 \times 10^7$                        | 1/6                  | 0/10           | 4/10                    | 2/10                    | 3/10                     | 3/10                     |
| $1.64 \times 10^6$                        | 0/7                  | 0/10           | 1/10                    | 2/10                    | 1/10                     | 1/10                     |
| LD <sub>50</sub> and 20 x 10 <sup>6</sup> |                      |                | 50 x 10 <sup>6</sup>    | 100 x 10 <sup>6</sup>   | 25 x 10 <sup>6</sup>     | 16 x 10 <sup>6</sup>     |
| 95% (2.0 x 10 <sup>6</sup> )              |                      |                | (11 x 10 <sup>6</sup> ) | (25 x 10 <sup>6</sup> ) | (5.5 x 10 <sup>6</sup> ) | (3.2 x 10 <sup>6</sup> ) |
| Confidence to                             |                      |                | to                      | to                      | to                       | to                       |
| Limits 200 x 10 <sup>6</sup> )            |                      |                | 225 x 10 <sup>6</sup> ) | 400 x 10 <sup>6</sup> ) | 112 x 10 <sup>6</sup> )  | 80 x 10 <sup>6</sup> )   |
| Agglutinin<br>titer                       | 0                    | 1:32           | 0                       | 1:16                    | 0                        | 1:16                     |

Table V. Effect of chronic cold stress (2° C) on mortality of mice challenged with Staphylococcus aureus, strain Fritchie. Animals maintained for 21 days, immunized and challenged at noted temperatures. \*7 day mortality (dead/total).

experiments, acclimatized and immunized mice, singly-caged or in groups, did no better on challenge with S. aureus than controls.

## Low Ambient Temperatures and Specific and Nonspecific Resistance

Effect of acute exposure following one LD<sub>50</sub> challenge dose. The animals in this experiment were maintained at 21° C during the time of specific immunization or treatment with zymosan or endotoxin. Following challenge with viable organisms, some of the animals were immediately transferred to 2° C; others were kept at 21° C as controls. The mice that were transferred to the cold room were either kept in groups of 10 or caged indi-

MIYA, MARCUS AND PHELPS

| Temperature | Treatment | Mortality ratios at 14 days |          |
|-------------|-----------|-----------------------------|----------|
|             |           | (grouped)                   | (single) |
| 21° C       | Normal    | 8/10                        | -        |
|             | Immune    | 1/10                        | -        |
|             | Zymosan   | 6/10                        | -        |
|             | Endotoxin | 9/10                        | -        |
| 2° C        | Normal    | 10/10                       | 8/10     |
|             | Immune    | 1/10                        | 4/10     |
|             | Zymosan   | 8/10                        | 9/10     |
|             | Endotoxin | 7/10                        | 9/10     |
|             | -         | 0/10                        | 1/10     |

Table VI. Effect of acute cold (2° C) on mortality of mice challenged intraperitoneally with 1 LD<sub>50</sub>\* of Staphylococcus aureus, strain Fritchie. Unacclimatized animals immunized or treated and challenged at room temperature, then placed at noted temperatures. \*1 LD<sub>50</sub> of S. aureus = 75 x 10<sup>6</sup> organism per mouse as determined by method of Litchfield and Wilcoxon (1949).

vidually. The mortality ratios that were obtained under these conditions are shown in Tables VI and VII.

Table VI shows that mice challenged with 1 LD<sub>50</sub> of S. aureus are afforded protection when specifically immunized and kept in groups of ten while acutely exposed to 2° C. However, mice caged as individuals are not afforded the same degree of protection, although resistance greater than that of normal mice is evident. The nontreated acutely stressed control mice did not die when grouped, and only 10 per cent (1/10) died when caged singly. Therefore, most of the deaths that occurred may be attributed primarily to the challenge of organisms. Mice receiving zymosan or endotoxin treatment prior to challenge did not fare as well as the specifically immunized animals, and the significance of the difference in mortality ratios between the normal and nonspecifically immunized



# SPECIFIC AND NONSPECIFIC RESISTANCE

| Temperature | Treatment | Mortality ratios at 14 days<br>(grouped) | (single) |
|-------------|-----------|--|----------|
| 21° C       | Normal    | 3/10                                     | -        |
|             | Immune    | 0/10                                     | -        |
|             | Zymosan   | 0/10                                     | -        |
|             | Endotoxin | 2/9                                      | -        |
| 2° C        | Normal    | 3/10                                     | 2/10     |
|             | Immune    | 0/10                                     | 4/10     |
|             | Zymosan   | 3/10                                     | 4/10     |
|             | Endotoxin | 4/10                                     | 7/10     |
|             | -         | 0/10                                     | 1/10     |

Table VII. Effect of acute cold (2° C) on mortality of mice challenged intraperitoneally with 1 LD<sub>50</sub>\* of *Klebsiella pneumoniae*. Unacclimatized animals immunized or treated and challenged at room temperature, then placed at noted temperatures. \*1 LD<sub>50</sub> of *K. pneumoniae* = 68 organisms per mouse as determined by method of Litchfield and Wilcoxon (1949).

mice is not significant. It should be mentioned here that mice kept at 21° C in groups or caged singly did not show any differences in mortality ratios as determined by preliminary experiments, and therefore the results obtained with the mice kept at 21° C in groups can serve as controls for the mice kept as individuals at 2° C.

The results presented in Table VII summarize the mortality ratios obtained when mice are subjected to acute cold stress following challenge with 1 LD<sub>50</sub> of *K. pneumoniae*. Again, specific immunization afforded the best protection against the induced infection when mice were subjected to acute cold stress. However, the mice kept in groups were better protected than mice caged individually and subjected to low ambient temperatures. The mice treated with zymosan and kept at 21° C showed no mortality, but the mice receiving the same treatment and placed at 2° C, whether



MIYA, MARCUS AND PHELPS

| Temperature | Treatment | Mortality ratios at 14 days<br>(grouped) | (single) |
|-------------|-----------|--|----------|
| 21° C       | Normal    | 8/10                                     | -        |
|             | Immune    | 1/10                                     | -        |
|             | Zymosan   | 6/10                                     | -        |
|             | Endotoxin | 9/10                                     | -        |
| 2° C        | Normal    | 9/10                                     | 10/10    |
|             | Immune    | 0/10                                     | 2/10     |
|             | Zymosan   | 6/10                                     | 8/10     |
|             | Endotoxin | 7/10                                     | 7/10     |
|             | -         | 0/10                                     | 0/10     |

Table VIII. Effect of chronic cold stress (2° C) on mortality of mice challenged intraperitoneally with 1 LD<sub>50</sub>\* of Staphylococcus aureus, strain Fritchie. Animals maintained for 30 days, immunized or treated and challenged at noted temperatures.  
\*1 LD<sub>50</sub> of S. aureus = 75 x 10<sup>6</sup> organisms per mouse.

in groups or singly, were not afforded this protection. The results obtained with endotoxin are equivocal. The mortality ratio of the normal animals kept at 21° C was only 30 per cent (3/10). Results to be presented later in this paper deal with mortality ratios obtained when the challenge dose was increased to 10 LD<sub>50</sub>.

Effect of chronic exposure following one LD<sub>50</sub> dose. The animals in this experiment were maintained at 2° C for 30 days in order to allow acclimatization to the low ambient temperature. The animals were specifically immunized or treated with zymosan or endotoxin at this low ambient temperature. Following these procedures the animals were challenged with one LD<sub>50</sub> dose of S. aureus or K. pneumoniae. The results are shown in Tables VIII and IX.

The animals receiving the S. aureus challenge (Table VIII) were protected if they were specifically immunized. Such protection occurred without regard to whether the mice were caged in groups

# SPECIFIC AND NONSPECIFIC RESISTANCE

| Temperatures | Treatment | Mortality ratios at 14 days |          |
|--------------|-----------|-----------------------------|----------|
|              |           | (grouped)                   | (single) |
| 21° C        | Normal    | 3/10                        | -        |
|              | Immune    | 0/10                        | -        |
|              | Zymosan   | 0/10                        | -        |
|              | Endotoxin | 2/9                         | -        |
| 2° C         | Normal    | 4/10                        | 6/10     |
|              | Immune    | 0/10                        | 2/10     |
|              | Zymosan   | 0/10                        | 9/10     |
|              | Endotoxin | 9/10                        | 7/10     |
|              | -         | 0/10                        | 0/10     |

Table IX. Effect of chronic cold stress (2° C) on mortality of mice challenged intraperitoneally with 1 LD<sub>50</sub>\* of Klebsiella pneumoniae. Animals maintained for 30 days, immunized or treated and challenged at noted temperatures. \*1 LD<sub>50</sub> of K. pneumoniae = 68 organisms per mouse.

or as individuals. The nonspecific immunization treatment did not result in significant protection compared to the controls or to the specifically immunized animals. The mortality ratio of the zymosan treated animals compared to the endotoxin treated animals is not significantly different. When comparing mortality results of animals that are acclimatized versus the acutely cold stressed animals (Table VI vs. Table VIII), note that the acclimatized animals that are specifically immunized did well grouped or caged individually. In contrast, the acutely cold stressed singly-caged mice were not benefited by specific immunization to the extent noted for the acutely stressed mice caged in groups.

When the cold acclimatized mice were challenged with one LD<sub>50</sub> of K. pneumoniae (Table IX), both specific immunization and zymosan treatment were beneficial to animals kept at 21° C or in groups at 2° C. However, protective effects of endotoxin were not apparent under these conditions. When animals that have been

MIYA, MARCUS AND PHELPS

| Temperature | Treatment | Mortality ratios at 14 days<br>(grouped) |
|-------------|-----------|--|
| 21° C       | Normal    | 10/10                                    |
|             | Immune    | 0/10                                     |
|             | Zymosan   | 9/10                                     |
|             | Endotoxin | 10/10                                    |
| 2° C        | Normal    | 10/10                                    |
|             | Immune    | 0/10                                     |
|             | Zymosan   | 9/10                                     |
|             | Endotoxin | 10/10                                    |
|             | -         | 0/10                                     |

Table X. Effect of acute cold stress (2° C) on mortality of mice challenged intraperitoneally with 10 LD<sub>50</sub>\* of Staphylococcus aureus, strain Fritchie. Unacclimatized animals immunized or treated and challenged at room temperature, then placed at noted temperatures. \*10 LD<sub>50</sub> of S. aureus = 50 x 10<sup>8</sup> organisms per mouse.

acutely cold stressed are compared to acclimatized animals, the latter do better than the former following challenge. It appears that zymosan may benefit the challenged host in the animals kept in groups under conditions of chronic cold stress, but not under conditions of acute cold stress. This protective effect of zymosan is not apparent in acclimatized animals caged singly. Again, some reservation in conclusions is warranted because of the low mortality ratio of the mice kept at 21° C following the one LD<sub>50</sub> challenge dose.

Effects of acute exposure following larger challenge dose. The specifically or nonspecifically treated animals in this experiment were maintained and challenged at room temperature. Following this, the animals were immediately transferred to 2° C or kept at 21° C as controls. The challenge dose was increased by several orders of magnitude over that in the previous experiments in order to obtain more definitive results concerning the efficacy of nonspecific versus specific resistance to disease. The animals were caged in groups of

# SPECIFIC AND NONSPECIFIC RESISTANCE

| Temperature | Treatment | Mortality ratios at 14 days<br>(grouped) | P(x <sup>2</sup> ) |
|-------------|-----------|--|--------------------|
| 21° C       | Normal    | 15/20                                    | 0.05 - 0.02        |
|             | Immune    | 0/20                                     |                    |
|             | Zymosan   | 8/20                                     | 0.3 - 0.2          |
|             | Endotoxin | 11/19                                    | 0.5 - 0.3          |
| 2° C        | Normal    | 17/20                                    | 0.02 - 0.01        |
|             | Immune    | 0/20                                     |                    |
|             | Zymosan   | 9/20                                     | 0.3 - 0.2          |
|             | Endotoxin | 14/20                                    | 0.2 - 0.1          |
|             | -         | 0/10                                     |                    |

Table XI. Effect of acute cold stress (2° C) on mortality of mice challenged intraperitoneally with 10 LD<sub>50</sub>\* of *Klebsiella pneumoniae*. Unacclimatized animals immunized or treated and challenged at room temperatures, then placed at noted temperatures. \*10 LD<sub>50</sub> = 1000 organisms per mouse.

ten. The results are shown in Tables X and XI. Table X shows that mice specifically immunized to the challenge agent, *S. aureus*, were significantly protected even though acutely exposed to 2° C. The mortality ratios of mice treated with zymosan or endotoxin prior to challenge was considerably increased over the specifically immunized group. The results indicate that the increased resistance afforded mice by specific immunization was not depressed by acute exposure of the animals to 2° C. The resistance induced, then, appeared to be temperature independent. There were no singly-caged animals in this experiment, since it was apparent from prior results that grouping afforded maximal protection even in groups with as few as three animals.

In Table XI are summarized the results of acute cold stress of specifically and nonspecifically immunized animals challenged with ten LD<sub>50</sub>'s of *K. pneumoniae*. By increasing both the animal group size and challenge inoculum dose, we felt that the data obtained would



## MIYA, MARCUS AND PHELPS

| Temperature | Treatment | Mortality ratios at 14 days<br>(grouped) |
|-------------|-----------|--|
| 21° C       | Normal    | 10/10                                    |
|             | Immune    | 0/10                                     |
|             | Zymosan   | 9/10                                     |
|             | Endotoxin | 10/10                                    |
| 2° C        | Normal    | 10/10                                    |
|             | Immune    | 1/10                                     |
|             | Zymosan   | 10/10                                    |
|             | Endotoxin | 10/10                                    |
|             | -         | 0/10                                     |

Table XII. Effect of chronic cold stress (2° C) on mortality of mice challenged intraperitoneally with 10 LD<sub>50</sub>\* of *Staphylococcus aureus*, strain Fritchie. Animals maintained for 30 days, immunized or treated and challenged at noted temperatures. \*10 LD<sub>50</sub> of *S. aureus* = 50 x 10<sup>8</sup> organisms per mouse.

represent definitive results concerning whether nonspecific immunization procedures were as efficacious as specific immunization procedures. And once more, specific immunization offered the greatest resistance to experimental disease. This increased resistance was not affected by acute exposure to 2° C. The extent of protection afforded the animals by treatment with zymosan or endotoxin did not approach that afforded by specific immunization. By comparing the mortality ratio of the control animals with that of the zymosan treated animals, a probability value of significance is obtained. This significant degree of protection was independent of acute cold exposure. No significant differences in mortality ratios were noted between control animals and endotoxin treated animals.

Effect of chronic exposure following larger doses. Animals in this experiment were maintained at 2° C for 30 days to allow acclimatization. The animals were specifically or nonspecifically immunized at this low ambient temperature, and were caged in groups of 10. The challenge dose was increased to several LD<sub>50</sub>'s in order to obtain



# SPECIFIC AND NONSPECIFIC RESISTANCE

| Temperature | Treatment | Mortality ratios at 14 days<br>(grouped) |
|-------------|-----------|--|
| 21° C       | Normal    | 10/10                                    |
|             | Immune    | 0/10                                     |
|             | Zymosan   | 3/10                                     |
|             | Endotoxin | 8/10                                     |
| 2° C        | Normal    | 10/10                                    |
|             | Immune    | 0/10                                     |
|             | Zymosan   | 1/10                                     |
|             | Endotoxin | 10/10                                    |
|             | -         | 0/10                                     |

Table XIII. Effect of chronic cold stress (2° C) on mortality of mice challenged intraperitoneally with 10 LD<sub>50</sub>\* of Klebsiella pneumoniae. Animals maintained for 30 days, immunized or treated and challenged at noted temperatures. \*10 LD<sub>50</sub> of K. pneumoniae = 1000 organisms per mouse.

more definitive results concerning the efficacy of specific and non-specific immunization under these conditions. Table XII shows the results obtained for animals challenged with S. aureus, and it is evident that chronic cold stress did not interfere with specific immune processes. Specifically immunized animals were significantly protected against parenterally induced disease even when exposed to challenge doses as high as 10 LD<sub>50</sub> 's. There was no protection afforded the animals pretreated with zymosan or endotoxin. It should be mentioned here that the strain of S. aureus employed caused the deaths of the animals within 24 hours post-challenge. Those animals surviving the first 24 hours usually did not die subsequently. Therefore, the experiments were terminated at 14 days.

Table XIII shows the results of chronically cold exposed animals challenged with K. pneumoniae. Again, as noted in experiments already described, the specifically immunized animals were significantly protected against the ten LD<sub>50</sub> challenge. Endotoxin treatment yielded no protection to the mice. In contrast, the zymosan treated

animals had only 1 death in 10 at 2° C and only 3 deaths in 10 at 21° C. The results obtained with zymosan pretreatment are quite similar to those obtained with mice acutely cold stressed following challenge with K. pneumoniae. This would suggest that the mechanisms whereby zymosan acts to increase resistance to experimental disease is independent of acute or chronic exposure at 2° C.

### Viruses

Coxsackie virus infections are manifest in different organ systems in infant mice, but not in adult mice (Dalldorf, 1950; Pappenheimer, Kunz, and Richardson, 1951; Boring, Angevine, and Walker, 1955). Since the morbidity and mortality properties of the Coxsackie strains obtained for these experiments was not known, preliminary experiments were designed to select the strain most suitable for experiments with adult mice, and the results of this screening procedure are summarized in Table XIV, which shows that challenge with Type B-3 Coxsackie virus caused mortality whether mice were kept at 2° C or 21° C. The University of Utah Type B-1 strain caused 4 deaths in 4 at 2° C and 1 death in 4 at 21° C, whereas the Connecticut 5 strain of Type B-1 caused 2 deaths in 4 at 2° C and zero deaths in 4 at 21° C. Since the experimental program as planned required a viral agent that would cause death of challenged animals at one temperature but not at the other, it was decided that the Type B-1 virus strain would be further screened with respect to mortality enhancement. The results shown in Table XV point out that neither viral agent caused mortality in mice kept at 21° C; however, a significant difference in mortality ratios is seen in mice kept at 2° C. The University of Utah Type B-1 Coxsackie virus strain was chosen as the subsequent experimental agent.

In the first definitive experiment, mice were acclimatized at 2° C for 40 days prior to challenge. The animals were to be compared with unacclimatized animals and control animals kept at 21° C. The mice were randomly segregated into groups often prior to challenge. Mice were pretreated with either zymosan or endotoxin. In addition a third group was given formalin killed virus 7 days prior to challenge. Unacclimatized mice were kept, treated, challenged at 21° C, and then placed at 2° C. The protocol and results are

# SPECIFIC AND NONSPECIFIC RESISTANCE

| Virus Strain    | Mortality ratios at 14 days*** |       |
|-----------------|--------------------------------|-------|
|                 | 2° C                           | 21° C |
| B-1*            | 4/4                            | 1/4   |
| B-2*            | 1/4                            | 0/4   |
| B-3*            | 4/4                            | 3/4   |
| B-4*            | 1/4                            | 0/4   |
| B-5*            | 1/4                            | 0/4   |
| B-1 (Conn. 5)** | 2/4                            | 0/4   |
| Cold Control    | 0/4                            | -     |

Table XIV. Comparison of different Type B Coxsackie virus strains on mortality production in unacclimatized adult mice. \*University of Utah, Department of Microbiology Stock Strains. \*\*Obtained from Dr. D. L. Walker. \*\*\*All challenge doses approximately  $5 \times 10^4$  PFU i. p.

| Virus                   | Mortality ratios at 14 days* |       |
|-------------------------|------------------------------|-------|
|                         | 2° C                         | 21° C |
| University of Utah, B-1 | 19/20                        | 0/20  |
| Conn. 5, B-1            | 9/20                         | 0/20  |

Table XV. Mortality ratios of unacclimatized adult mice challenged with Type B-1 Coxsackie virus. \*Challenge dose =  $5 \times 10^4$  PFU i. p.

| Temperature                   | Treatment    | Mortality ratios at 7 days |
|-------------------------------|--------------|----------------------------|
| 21° C                         | Normal       | 0/10                       |
|                               | Immunized    | 0/10                       |
|                               | Zymosan      | 0/10                       |
|                               | Endotoxin    | 1/9                        |
| 2° C<br>(Unacclima-<br>tized) | Normal       | 7/10                       |
|                               | Immunized    | 0/10                       |
|                               | Zymosan      | 3/10                       |
|                               | Endotoxin    | 8/8                        |
|                               | Cold Control | 0/10                       |
| 2° C<br>(Acclimatized)        | Normal       | 0/10                       |
|                               | Immunized    | 0/10                       |
|                               | Zymosan      | 2/10                       |
|                               | Endotoxin    | 3/10                       |
|                               | Cold Control | 0/10                       |

Table XVI. Effect of specific and nonspecific immunization on resistance of adult mice challenged with Type B-1 Cocksackie virus intraperitoneally. Challenge =  $10 \times 10^4$  PFU.

summarized in Table XVI. The mortality ratios were taken only for 7 days post-challenge because the mice were inadvertently not fed one weekend, and most of the animals in the cold died, presumably from starvation. However, it is of interest to briefly analyze the results obtained. It is seen that the virus only caused one death in 39 mice kept at 21° C. This death occurred in the animals receiving endotoxin. It is also seen that unacclimatized mice did not fare as well as acclimatized mice and, in general, exposure to 2° C decreased the host's ability to withstand Cocksackie virus challenge. Of special interest is the indication that acclimatized mice seemed to withstand the Type B-1 Cocksackie virus challenge that was detrimental to unacclimatized mice, although specific immunization

# SPECIFIC AND NONSPECIFIC RESISTANCE

| Temperature                   | Treatment    | Mortality ratios at 14 days |
|-------------------------------|--------------|-----------------------------|
| 21° C                         | Normal       | 0/20                        |
|                               | Immunized    | 0/20                        |
|                               | Zymosan      | 0/20                        |
|                               | Endotoxin    | 3/20                        |
| 2° C<br>(Unacclima-<br>tized) | Normal       | 15/20                       |
|                               | Immunized    | 0/20                        |
|                               | Zymosan      | 8/20                        |
|                               | Endotoxin    | 20/20                       |
|                               | Cold Control | 0/20                        |
| 2° C<br>(Acclimatized)        | Normal       | 1/20                        |
|                               | Immunized    | 0/20                        |
|                               | Zymosan      | 4/20                        |
|                               | Endotoxin    | 7/20                        |
|                               | Cold Control | 0/20                        |

Table XVII. Coxsackie B-1 infection mortality in adult mice. Challenge =  $10 \times 10^4$  PFU intraperitoneally.

procedures protected the animals under these conditions. With the doses and routes of administration employed in this experiment, zymosan and endotoxin did not enhance host resistance to viral disease.

Although most deaths of animals in the preliminary experiment occurred between 4 and 7 days post-challenge and probably the results of the first definitive experiments were valid at 7 days, further investigation was required in order to see if acclimatization was beneficial to the challenged animals. Therefore, a repeat experiment of the same design was completed, but larger groups of mice were employed. The animals kept at 2° C were supplied with adequate amounts of food and water to rule out nutritional factors that might



confuse the analysis of data. The results of the experiment are shown in Table XVII. The majority of deaths occurred between 4 and 6 days post-challenge; no deaths of animals occurred after 10 days following challenge. Again, acclimatization at 20° C for 40 days resulted in mortality ratios similar to those observed in the animals kept at 21° C. Unacclimatized mice had increased mortality ratios in all treated groups compared to the 21° C control mice except in the immunized groups. Zymosan appeared to give greater protection than endotoxin; in fact, the 20 out of 20 deaths in the endotoxin group occurred by the fourth post-challenge day. If the mice were acclimatized and treated with endotoxin, only 7 in 20 deaths occurred. Thus, it would appear that acclimatization enhances host resistance to challenge in addition to decreasing the increased mortality ratios in the groups treated with endotoxin. The value of specific immunization is apparent in the unacclimatized mice.

#### Low Ambient Temperature and Ehrlich Ascites Tumor

The effect of temperature on neoplastic diseases has been studied by various investigators (Fay and Henny, 1938; Smith and Fay, 1939; Bischoff and Long, 1939; Wallace et al., 1944; Fuller et al., 1941; Goldfeder, 1941; Wallace et al., 1942; Tannenbaum and Silverstone, 1949; Griffiths et al., 1961). Effects on the tumor take, incidence of spontaneous tumor formation, growth, or regression were noted as a result of high or low ambient temperatures, but no consistent effects have been noted. In this respect, the effect of temperature on neoplastic disease becomes as complicated as that observed with regard to bacterial or viral disease.

Since the Ehrlich ascite tumor produces certain effects in mice similar to infectious disease, for example, rapidly progressive disease, it was chosen as a model to study the effect of cold stress on the disease process. Mice were exposed in the cold room in large animal cages containing 50 mice and acclimatized to this temperature for 45 days. Two days prior to challenge the mice were caged in groups of six. The mice were given unlimited amounts of Purina mouse chow and tap water. Mice kept at room temperature were randomly separated into groups of 12.

# SPECIFIC AND NONSPECIFIC RESISTANCE

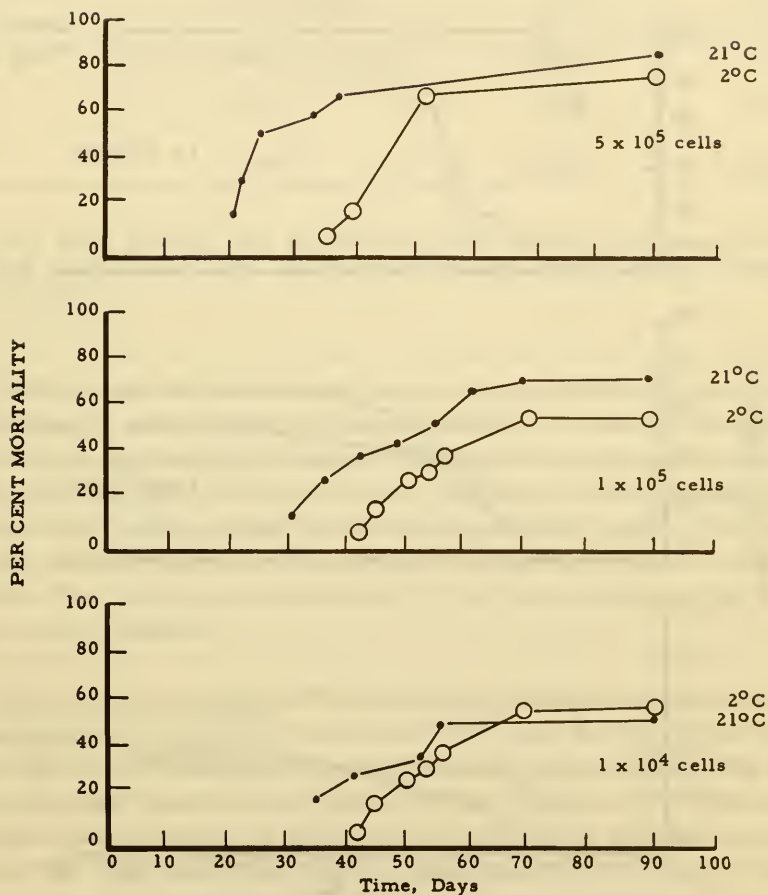


Figure 13. Mortality curves of acclimatized mice subcutaneously challenged with Ehrlich Ascites tumor cells and maintained at low ambient temperature (2° C).

# MIYA, MARCUS AND PHELPS

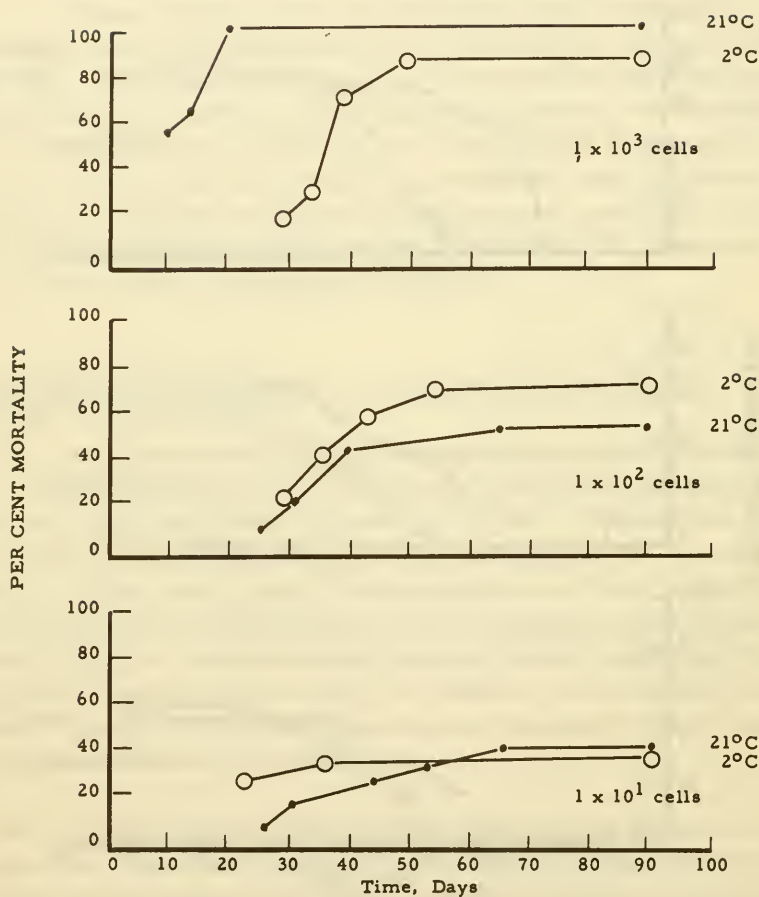


Figure 14. Mortality curves of acclimatized mice intraperitoneally challenged with Ehrlich Ascites tumor cells and maintained at low ambient temperature (2°C).

# SPECIFIC AND NONSPECIFIC RESISTANCE

| Route   | Inoculation<br>Dose | Mortality Ratios |       |
|---------|---------------------|------------------|-------|
|         |                     | 2° C             | 21° C |
| S. C.*  | 10 <sup>4</sup>     | 4/12***          | 6/12  |
|         | 10 <sup>5</sup>     | 7/12             | 9/12  |
|         | 5 x 10 <sup>5</sup> | 9/12             | 9/12  |
| I. P.** | 10                  | 4/12             | 6/12  |
|         | 100                 | 7/12             | 8/12  |
|         | 1000                | 11/12            | 12/12 |

Table XVIII. Mortality ratio of acclimatized mice kept at 2° C and inoculated with Ehrlich Ascites tumor cells. \*Subcutaneous Challenge. \*\*Intraperitoneal Challenge. \*\*\*Dead/Total.

Ehrlich ascites tumor cells were kept in a stock suspension and occasionally were passed intraperitoneally into mice. The tumor cells were harvested and washed three times with Hank's balanced salt solution (BSS). The cells were suspended in Hank's BSS, and viable cells were counted in the hemocytometer using a 0.05 per cent solution of eosin as diluent. Stained cells were considered non-viable. The cells were then diluted to the desired concentration in the same medium.

Subcutaneous challenge. The challenge inocula were prepared as described and adjusted to contain 10<sup>4</sup>, 10<sup>5</sup>, and 5 x 10<sup>5</sup> tumor cells per 0.5 ml. The cells were injected into the nuchal region. The experiment was terminated 90 days after inoculation of the cells. Low ambient temperatures as shown in Table XVIII did not significantly affect the final mortality ratios, regardless of the inoculum size. However, when animal mortality is plotted as a function of time, a significant delay in mortality is seen (Fig. 13). The effect is most pronounced in the highest challenge doses while a graded diminished effect is evident in the lower doses.

Intraperitoneal challenge. The challenge inocula were prepared as described and adjusted to contain 10, 100, and 1000 tumor cells per 0.5 ml. The acclimatized mice were challenged intraperitoneally,

and the mortality of the animals was followed for 90 days after challenge. The results in Table XVIII show that again low ambient temperatures did not influence the final mortality ratios regardless of challenge dose. However, when mortality is plotted as a function of time, a significant delay in mortality again is seen (Fig. 14). This delay in mortality is most pronounced in the animals receiving the highest challenge dose. At lower challenge doses, the mice died more rapidly when kept at 2° C than when kept at 21° C.

## DISCUSSION

At the onset of this investigation, it was felt that knowledge of the metabolic behavior of the bacterial challenge agents was important in order to assess properly any subsequent data obtained when these agents were employed. The experimental results indicated that temperatures lower than 37° C affected the oxygen uptake but not the generation time of the organisms. The only apparent effect of lowered temperatures employed was to increase the lag phase.

Along with the above information, the necessity to determine the effect of low ambient temperatures on the body temperature of the mice used in our experimental set-up became apparent. As a first approximation, we assumed that if the body temperature changed as a result of cold exposure, then the change could directly influence the course of the experimental disease by enhancing or depressing the ability of the organisms to grow and develop. Our results showed that the rectal temperature of mice exposed to 2° C fluctuated within the first 24 hour period of cold exposure, but stabilized thereafter at a temperature 1° C to 2° C higher than mice kept at room temperature. The fluctuation was more pronounced in mice caged singly as compared to those caged in groups of five. In contrast, Walker and Boring (1958) showed that rectal temperatures of mice exposed to 4° C decreased approximately 1° C. The differences between results of the two groups reporting might be ascribed to a significant technical detail; that is, we in-



## SPECIFIC AND NONSPECIFIC RESISTANCE

serted the probe uniformly 2 cm into the rectum, whereas Walker and Boring inserted the probe only 1 cm.

In spite of the differences in results of these two groups with regard to the increase or decrease in the body temperature of mice as a result of cold exposure, the degree of change was not outside the range compatible for optimal growth and development of the organisms employed for parenteral challenge. Therefore, we felt that any changes in susceptibility or resistance to disease could now be ascribed to factors other than that related to core temperature per se.

The fact that the K. pneumoniae isolated from animals maintained at 2° C did not vary in virulence as compared to the same organism isolated from mice maintained at 21° C led us to conclude that this organism could be used with confidence as a challenge agent to obtain the desired mortality ratios when given to mice kept at 21° C or 2° C. The observation that the isolate from mice maintained at 2° C would grow equally well at incubation temperatures of 37° C or 32° C requiring only one-half the oxygen used at 32° C suggests that these organisms are more metabolically efficient in contrast to organisms isolated from animals maintained at 21° C and grown under the same conditions. This deserves further investigation.

In general, specific immunization protected significantly, whereas nonspecific substances which have been used to increase resistance (zymosan and endotoxin) did not afford the same degree of protection when the challenged animals were exposed to 2° C either as acclimatized or unacclimatized mice. This was true whether the challenge agent was K. pneumoniae, S. aureus, or the B-1 strain of Coxsackie virus.

The increased resistance afforded by immunization was maximal when mice were exposed to low ambient temperatures in groups. Mice that were caged individually did not fare so well. Since the core temperatures of mice caged individually or in groups equilibrated within 24 hours of low temperature exposure, one cannot validly state that loss of body heat is a contributing factor to lessened resistance. However, it might be hypothesized that the metabolic rate of individually caged mice is increased over that of grouped animals,

since there is opportunity for greater heat loss and this increased metabolism is sufficient to result in the same end body temperature. Further, this increased metabolic rate resulting from the low temperature stress might eventually lead to exhaustion of body reserves and subsequent death (Selye, 1950).

Another possibility to account for variations in results is a psychological factor. Since immunized non-challenged mice are able to survive low ambient temperatures when caged individually but not when challenged with an infectious disease agent and maintained individually at low ambient temperatures, one must speculate concerning the extent that the factor of isolation contributes to increased mortality observed. Psychological factors cannot be disregarded in assessing this problem.

In some instances, zymosan or endotoxin treatment resulted in increased mortality ratios. This was noted consistently when the challenge agent was K. pneumoniae or the strain of Coxsackie virus, but not as apparent when S. aureus was used. This paradox, if real, requires further investigation, since nonspecific immunizing agents are known either to enhance or depress resistance depending on the time of administration; yet our observations suggest that the dosage and administration time optimal for a given ambient temperature may not be optimal for another ambient temperature.

The relationship of zymosan to properdin, shown to exist by Pillemer et al. (1956), deserves attention with regard to speculation concerning the extent the properdin system plays in the observed results with zymosan in our experiments. The dosage of 9 mgm subcutaneously is in excess of that reported by Ross (1956) to stimulate increases in properdin levels in mice. Ross injected the material intravenously, and the colloidal nature of zymosan certainly would limit the amount employed in order to avoid pulmonary embolic complications. Recently, Iakovleva and Remezov (1960) reported that mice exposed to the cold have greater than normal levels of properdin 72 hours post-exposure. The properdin level increasing action of zymosan added to the levels obtainable by cold exposure should insure mice of a high properdin level. However, since the serum of mice lacks complement components (Rice and Crowson, 1950) and has been shown to be devoid of bactericidal activity

## SPECIFIC AND NONSPECIFIC RESISTANCE

(Marcus, Esplin and Donaldson, 1954), it seems unlikely that the properdin system is contributing much to the natural defenses of the mouse (Miya, Marcus, and Perkins, 1960).

Results of our experiments indicate that mice chronically exposed to cold are able to form agglutinin antibody. Trapani (1960) reported that cold exposed rabbits were able to form antibody almost as rapidly as rabbits kept at room temperature. In contrast, Ipsen (1952) reported that antibody formation is impaired in mice exposed to 4° C. We have not attempted to determine if antibody formation is impaired by acute exposure to cold.

Although the results of the Cocksackie virus experiments suggest that acclimatization will result in a normal degree of host resistance of nonimmune animals against challenge with this agent, the results of others are not in agreement. Walker and Boring (1958) reported on experiments using a Connecticut 5 strain of Type B-1 Cocksackie virus. They observed that acute limited exposure to 4° C was insufficient to change the viral infection from an asymptomatic into a lethal process, but that continued exposure for several days did accomplish conversion to disseminated lethal disease. They reported on experiments in which adapted (14 days) animals were employed, and found that this period of adaptation at 4° C did not counteract the lethal effects of the virus disease at 4° C. Mice challenged at 4° C, following acclimatization and then placed at 25° C, did not die. Therefore, Walker and Boring concluded that exposure to 4° C caused a decrease in resistance of the challenged animals.

In our experiments we have shown that acclimatization for 40 days at 2° C is sufficient for the adult mice to overcome a challenge dose that is lethal for virus challenged unacclimatized mice replaced in the cold box. Since the adrenal activity does initially increase upon cold exposure (Heroux and Hart, 1954; Schonbaum, 1960), and since the Cocksackie disease process resembled that due to cortisone effects, Walker and Boring (1958) attempted to reproduce the disease in mice at room temperature by ACTH injections following challenge with Cocksackie virus. They were unable to detect any decrease in resistance in mice treated in this manner. Although the increased mortality correlates well with increased corticosteroid



production following cold exposure, the exact cause and effect relationship remains obscure.

At present we are investigating the possibility that the virus may be present but inactive as a result of the acclimatization, but have the potential to cause disease or death if the challenged animal is removed from  $2^{\circ}\text{C}$  to  $21^{\circ}\text{C}$ . The situation could be analogous to that observed by Sulkin et al. (1960) with regard to bat rabies virus.

A brief discussion of the experiments with the mouse neoplasm is in order. Goldfeder (1941) reported that the environmental temperature has a definite effect on the growth rate but not the viability of subcutaneously inoculated tumor cells. Our results are similar in this respect. Although a delay in mortality occurred, no significant differences in the final mortality ratios were observed when mice exposed to cold were compared to mice challenged and kept at  $21^{\circ}\text{C}$ . An obvious working hypothesis is that the lowered skin temperature of mice kept at  $2^{\circ}\text{C}$  inhibited tumor cell metabolism until the tumor cells became acclimatized to the lower temperature. Once this occurred, it may be guessed, the growth rate of the acclimatized tumor cells was the same as for the controls.

The same temperature effect may be used to explain the delay in mortality of mice inoculated intraperitoneally; that is, the core temperature of  $2^{\circ}\text{C}$  exposed mice is about  $2^{\circ}\text{C}$  higher than mice kept at  $21^{\circ}\text{C}$ . This increased internal temperature may either alter tumor cell metabolism or allow for selection of tumor cells capable of growth at the new temperature. Work is continuing on this aspect of low ambient temperature and host resistance.

## SPECIFIC AND NONSPECIFIC RESISTANCE

### SUMMARY

In a series of experiments with mice, designed to explore the effect of low ambient temperatures on host-parasite relations, the following observations have been made:

1. The core temperature of mice exposed to 2° C stabilized within 24 hours 1° C to 2° C higher than that of mice maintained at 21° C.

2. The bacterial challenge agents, K. pneumoniae and S. aureus, were shown to be capable of growth at the core temperature fluctuations.

3. The K. pneumoniae employed was passed through and isolated from mice maintained at 2° C. This organism was equally capable of growth at incubation temperatures of 32° C or 37° C, although the oxygen requirements were one-half that required at 37° C.

4. Specific immunization resulted in the best protection when compared to zymosan or endotoxin treatment.

5. Zymosan and endotoxin treatment often resulted in increased mortality in mice exposed to 2° C and challenged with bacterial or viral agents.

6. Grouping of mice exposed to 2° C was significantly beneficial to survival following challenge as compared to singly-caged mice.

7. Acclimatization to 2° C resulted in equivocal protection to mice challenged with bacterial agents but was beneficial to mice challenged with a Coxsackie virus strain.

8. Low ambient temperatures influence the course of Ehrlich Ascites tumor disease process by retardation of mortality but do not affect the over-all mortality ratio.



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## SPECIFIC AND NONSPECIFIC RESISTANCE

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## SPECIFIC AND NONSPECIFIC RESISTANCE

### DISCUSSION

MIRAGLIA: You made a statement that the rectal temperature of the mouse equilibrates after 24 hours and then establishes a plateau that is  $1^{\circ}\text{C}$  higher than the temperature the animal started out with. We found the same thing in the first 24 hour period, but our plateau temperature has been approximately the normal temperature of the mouse, rather than  $1^{\circ}\text{C}$  higher. I was wondering if a possible explanation of this isn't the fact that animals, in our experience, anyway, lose their tails and ears after 14 to 21 days in the cold, and losing this cooling surface causes them to have a slight elevation in temperature. I noticed in Figure 9 that you kept your animals at  $2^{\circ}\text{C}$  for 30 days. Do you do this in all of your experiments?

MIYA: Our acclimatization period varies. At the present time, we are trying to establish some criterion for acclimatization and experiments are being conducted at the present time in which we are measuring the adrenal weight and animal weight. We intend to do eosinophil counts and we have even thought of checking for stress lymphocytes; I don't have any conclusive data yet, but I feel, based on what I have read in the literature, that if you used approximately thirty to forty or sixty days, this is considered in the opinion of most people to be an adequate acclimatization period.

MARCUS: How about the loss of tails and ears?

MIRAGLIA: Do your animals experience this? The animal loses the tail completely, in our experience, and I was wondering whether your animals do the same thing.

BERRY: Yours are grouped how?

MIYA: Five or ten together; at least five.

BERRY: With bedding?

MIYA, MARCUS AND PHELPS

MIYA: They have a slight amount of sawdust in the cage.

BERRY: This could make a difference.

MIYA: Our singly caged mice are placed in complete wire cages and do not have any bedding.

MIRAGLIA: Do these mice lose their tails?

MIYA: Occasionally you will see a singly caged mouse that will lose its tail completely.

PREVITE: Is your cage of a standard size, or intentionally smaller because you have only one mouse in it?

MIYA: The dimensions are approximately 4 in. by 4 in. by 7 in. per mouse.

PREVITE: He has plenty of room.

BERRY: After these animals have been acclimatized at low temperatures for forty-five days, have you subjected them to an even more acute exposure, say,  $-20^{\circ}$  C, in a deepfreeze, for example. Can they survive a super stress longer than animals that have not been subjected to this long period of acclimatization?

MIYA: We haven't done any experiments like that.

BERRY: This might be of value, because altitude exposed mice that have "acclimatized" are able to withstand a hypoxic level for longer periods of time than animals that have not been at simulated altitudes. Possibly these animals that have been maintained at a low temperature, say of  $2^{\circ}$  C, for some time, may be able to withstand more acute cold stress. If you could show something of this type, I would be a little happier about calling them acclimatized to cold. Your results afford a very good argument for acclimatization, because animals with prior experience at low temperature respond to challenge differently from animals that have never had a previous experience. One

## SPECIFIC AND NONSPECIFIC RESISTANCE

might be justified in calling them acclimatized, but are they acclimatized to cold? At least they have changed in such a way that their response to infection is different.

BLAIR: Did I understand you to say that the magnitude of reductions in oxygen uptake of the microorganisms at 32° C was about one-half of normal?

MIYA: One-half of that obtained at 37° C.

BLAIR: This is a rather striking bit of information. It really shakes me up a bit. We humans fancy ourselves as being the acme of biological development, and I don't know whether I am delighted or distressed to inform you that for the oxygen uptake in human beings at 32° C, the magnitude of reduction is exactly the same as it is with *Staphylococcus aureus*, so perhaps at least with regard to fundamental metabolism, we are not so different from our primitive brethren.

BERRY: Don't be surprised. I think this is a fundamental physiological fact. Some physiologists here would probably confirm that, wouldn't you, Dr. Vaughn? It is not surprising that a tissue subjected to any given temperature reduction would show approximately the same metabolic relationship.

MIRAGLIA: Have you found that the animals eat more when they are cold stressed?

MIYA: Yes, this was an inadvertant discovery. When we first started in this work, I didn't even know if the mouse could tolerate the cold, and on Friday, I put mice in the ice box and threw them a handful of food. On Monday, the mice were all uniformly dead, which I didn't quite understand. But this was the most reproducible thing. I noted that there was no food in the cage, and then I determined that they ate approximately five times that which they would normally eat at room temperature.

MIRAGLIA: I am very happy to hear this, because someone was wondering yesterday if cold was stress to the animal.

If food intake is any criterion, they certainly are stressed, because we have noticed the same thing.

SCHMIDT: In measuring the effect on conditioned organisms, you tested them after intraperitoneal injection at 32° C, yet rectal temperatures of your mice have been shown to be higher than those of the normal mouse after exposure to cold. I wonder why it is that you decided to investigate the characteristics of these organisms at a lower temperature? Why not investigate them at a higher temperature, because this is indeed what you have found in the experimental system.

MIYA: This was done when we first started into this work. We briefly scanned the literature and most of the reports indicated that there was a possible drop in rectal temperatures. We didn't have any suitable temperature measuring equipment at that time, so we decided to approach the problem from a back door; to find out what these organisms would do at different temperatures, anticipating this drop. However, we found quite to the contrary that the temperature elevated.

SCHMIDT: You haven't had the occasion yet to investigate the growth curves or other characteristics of these organisms at one or two degrees above the normal rectal temperature?

MIYA: No, we haven't.

REINHARD: In the experiments on the isolates of the "conditioned bacteria", how many isolates did you use from each group of mice for determination of oxygen uptake?

MIYA: These were pooled isolates.

REINHARD: How did you pool them?

MIYA: We killed the animals and then opened up the peritoneal cavities aseptically and just removed the exudate.

NUNGESTER: Without plating?



## SPECIFIC AND NONSPECIFIC RESISTANCE

MIYA: Yes, we removed the peritoneal exudate, pooled the samples, and reinjected this material into another group of animals.

MARCUS: And the data shown from the isolates would be organisms from the seventh pool.

REINHARD: How many determinations did you make of the oxygen requirements; how many parallel experiments did you run?

MIYA: Each Warburg flask was set up in duplicate, and the runs were done three times.

REINHARD: Isn't it possible you could have run into chance variations? I would be more comfortable if you said you ran fifty determinations.

MIYA: I could have shown a series of curves that would essentially show what I have shown here. I chose, rather than to confuse the issue, to show a group of typical curves obtained for any one determination.

REINHARD: Then how did you test these organisms after they had grown out again to see whether they recaptured their original state or whether they remained the same in their oxygen requirements?

MIYA: I don't think I quite understand.

NUNGESTER: He means, if they're carried along as a pure line culture, how long do they retain this altered characteristic?

MIYA: After the seventh passage, we did the oxygen uptake studies and we didn't do any more after that. We merely saved those organisms for the challenge in subsequent experiments.

WALKER: I'd just like to comment on this curious, interesting difference in the effect of cold on various strains of mice that Dr. Miraglia mentioned. In our strain of Wisconsin mice,



the body temperature is very easily changed, and this isn't a matter of how far you insert the probe into the rectum. We use one centimeter. We would go further, but we get a higher incidence of perforated colon and so on. This difference among mouse strains is fairly important, it seems to me, because I am prepared to propose that in virus infections, the change in body temperature is quite important, and I am really more impressed with the effect of the temperature of the tissues than with factors like stress. The effect of cold on viral infections has been quite variable, quite contradictory, and I wonder if the differences among not only species, but among strains of mice may not be partly responsible for this variability. As Dr. Campbell indicated, and as my experience has been, too, you can hardly change the body temperature of a rabbit, regardless of what you do. If you do go down low enough in environmental temperature, the temperature suddenly begins to change and it goes right on down and the animal quickly dies; but up to that point, you cannot really lower it. But in our experience, the mouse temperature is quite readily changed. If you raise the environmental temperature, you raise his body temperature.

MIYA: If you plot distance into the rectum on the abscissa and the temperature on the ordinate, you notice almost a linear relationship.

MARCUS: From two centimeters down to one?

MIYA: Well, we took it at .5, 1.0, 1.5, and 2.0 cm down.

PREVITE: I think this has also been confirmed by Halberg and Spink.<sup>1</sup> In my own experiences, the distance the probe was inserted made a significant difference in the rectal temperature recorded.

MARCUS: We have had no difficulty with rupturing. We haven't penetrated too many.

<sup>1</sup> Halberg, F., and W. W. Spink. 1956. Laboratory Investigation.

## SPECIFIC AND NONSPECIFIC RESISTANCE

PREVITE: Halberg and Spink advise  $23 \pm 2$  mm.

MIYA: Our mice here have been acutely exposed to cold with the rectal probe at 2 cm depth, and the temperature remains at this level, but at 1 cm depth, it falls. This is quite similar to the type of curves that you obtained.

WALKER: I have never measured body temperature over a brief period of time like that. I have always tested the amount of time it takes for the probe to come to a steady state, but I have not determined the temperature in the first few hours after exposure.

BLAIR: One final comment, if I may, about the accuracy of temperature measurement. This problem has been kicked around, of course, in large animals, and it has been demonstrated that the variations seem to occur during the dynamic changes in temperature, but once the temperature is stabilized, it really doesn't matter where you measure the so-called core temperature. I can't speak for the smaller animal, but certainly in the large animals it does make a difference of what the point of interest is. The changes occur physiologically and physically during the actual changes of temperature, but once the temperature is stabilized, it doesn't really matter where it's measured.

WALKER: Benzinger finds considerable difference between rectum and esophagus temperatures, as I recall, in the first few hours, but after that, it makes no difference.

BLAIR: So it depends upon what you are interested in studying.

WALKER: I am not quite clear as to whether or not the effect of cold on Cocksackie virus infection that you described required continued exposure to cold to get this increased mortality.

MARCUS: We didn't carry out any experiments that discontinued; in other words, the animals were put in the cold and they stayed to the continuation of the experiment, but there was no discontinuous effect as you studied.

MIYA, MARCUS AND PHELPS

WALKER: I was wondering about the difference between your mice and mine in readjusting their temperatures. That emphasizes the difference in mice, I believe.

MARCUS: Well, actually we are both using albino Mus musculus.

WALKER: Yes, but we are using a colony that we have that is originally derived from Webster-Swiss mice.

PREVITE: Are your housing conditions the same?

WALKER: Ours were in wire cages with metal bottoms and no bedding, but with five or six animals per cage.

PREVITE: No bedding? I think you mentioned using sawdust and something else. I had a question about rectal temperatures in acclimatized mice. It rather intrigues me in that you report the rectal temperatures are  $1^{\circ}\text{C}$  to  $2^{\circ}\text{C}$  higher 40 to 45 days after acclimatization.

MARCUS: No, not 45 days. This occurred within 24 hours. Dr. Miya showed a chart of the effect of measuring their temperatures as a function of the distance in the colon from a depth of 2 cm. He had points of 2.0, 1.5, 1.0, and .5 cm in the animal.

PREVITE: Does this persist, this elevated temperature, or is it something that is transient?

MARCUS: No, it persists and remains fairly steady.

PREVITE: This is what I mean. Once this temperature goes up, it stays up.

MARCUS: That is true,  $1^{\circ}\text{C}$  to  $2^{\circ}\text{C}$  higher than the normal, and this is with the probe uniformly inserted all the time.

PREVITE: Do you have any possible explanation? Why should the temperature go up? I have no idea why it would go up.

MIYA: As you probably know, there is a report, which I be-

## SPECIFIC AND NONSPECIFIC RESISTANCE

lieve Herrington<sup>2</sup> wrote which showed that the metabolic rates of mice increase linearly as the ambient temperature decreases. We feel that under these conditions, the "thermostat" has now been set at a new higher level in an attempt at maintenance of homeostatic conditions.

PREVITE: This animal, being a homeotherm, would maintain a constant body temperature regardless of the environmental temperature as long as it was able to.

TRAPANI: I am curious about one thing: Is the life span of the mouse, or any other animal for that matter, the same under these conditions of cold exposure as compared to the room temperatures? Our time relationships should be weighed if the life span is different.

MARCUS: I don't know what the life span of the mouse is if you put him in the cold.

PREVITE: Barnett and his group<sup>3</sup> have done work on this. They report that mice may be successfully reared in the cold. However, mice reared in the cold survive longer without nesting material than those transferred from a warm room and also deprived of cold. Selye<sup>4</sup> has shown extensive damage to critical organs can result from exposure of rats to cold.

MARCUS: Well, with these experiments that have gone up to three months involving animals in cold, you have discussed some of the modest changes that occur, at least they seem modest to me, in the periphery of the animal, and I am not aware of any gross changes that occur, any gross pathology that occurs in the animals that are autopsied which could be ascribed to cold alone. With regard to what you said, though, a moment ago, about the mouse being a homeothermic animal and you would expect it to maintain its temperature once it is stabilized at a fairly con-

<sup>2</sup> Am. J. Physiol. 129: 123. 1940.

<sup>3</sup> Barnett et al. 1959. Quart. J. Exp. Physiol. XLIV.

<sup>4</sup> Selye, H. 1943. Rev. Canad. Biol. 2.



stant level: now, I think this has been the experience that you have had, too.

PREVITE: My experiments were much shorter; they were carried out within a day or two.

MARCUS: Now, just a second. You said in a day or two. What happened in a day or two?

PREVITE: My results would agree in general with those that you presented for the first day. The only thing I am puzzled about is why, after it does stabilize, does it stabilize itself at a temperature  $2^{\circ}\text{C}$  or  $3^{\circ}\text{C}$  higher?

MARCUS: It is easy to guess about this. The animal in the cold is passing off a lot of heat, and it has to maintain its temperature. It eats five times or more as much as an animal does at room temperature. We never measured the metabolic rate involved, but it may be that it just sets this whole mechanism up.

VIERECK: Maybe this would explain some of the contradictory results. If you put an animal in the cold, it is good and bad for him at the same time. It is bad for him if it is a stress; consequently, if the animal is stressed by cold, he is suffering, and thus less well able to cope with other stresses such as infection; and at the same time, cold exposure could be considered as being good for the animal inasmuch as it does speed up metabolism. He is eating more, his oxygen consumption is higher, et cetera. Now, here is an idea: Maybe this general speeding up of metabolism includes protein metabolism. I don't know of any evidence for this one way or another, other than food intake studies. Now, if protein metabolism is speeded up, is it reasonable to suspect that antibody formation would be coupled with this and the synthesis of antibodies would be automatically in a higher gear after the animal has been in the cold? What do you think of that?

MARCUS: I discussed this with Dr. Trapani and he pointed out that Dr. Whipple, employing plasmaphoresis, showed that there was no protein that was as metabolically effective as gamma

## SPECIFIC AND NONSPECIFIC RESISTANCE

globulin for use as a metabolic thing. The point that I am trying to make is that antibody, as such, is not only a constituent of gamma globulin, but it is a part of a protein which can be employed by the animal as a source of amino acid for protein metabolism very nicely; and I am sure that under these circumstances, the turnover rate of protein in the animal is related to antibody production; at least I feel there must be a correlation here; however, I am certainly not an authority in that area.

CAMPBELL: We have shown that the half-life of protein decreased rather measurably under cold. There has been some preliminary work done which has been extremely interesting with tagged amino acids and tagged gamma globulin, and it would seem, as far as antibody protein goes, that in the normal synthesis, the body prefers amino acids to gamma globulin. When you start immunizing or bleeding, the body seems to prefer gamma globulin. So if you label these two things, in one situation, the amino acids will be incorporated; in the other case, the gamma globulin is broken down and reincorporated.

McCLAUGHRY: In relation to Dr. Previtte's question about homeothermia, I don't believe the temperature of a homeothermic animal is anywhere near as fixed as has been discussed here. As a matter of fact, Adolf<sup>5</sup> showed that if the conditions vary the thermostat may be re-set at a slightly different level, perhaps 1° C or 2° C from the original, after acclimatization. This varies in different physiological regulatory mechanisms, and I think that this has to be taken into account in the experiment that has been reported.

BERRY: Dr. Miraglia has some results related to this point. He was given some black mice from NIH and DBA, weren't they?

MARCUS: They have a variety of types; DBA, CBA.

BERRY: These mice were placed at 5° C. They were all dead within 24 hours. They were completely unable to withstand this

<sup>5</sup> Am. J. Physiol. 166: 62. 1951.

temperature. We were trying to compare mice of a different genetic makeup, but were defeated in the attempt.

MARCUS: Didn't we have a similar experience with some dark mice?

MIYA: We have not used black mice in any of our experiments.

BERRY: This shows the same difference in mice in a very dramatic way. The other question I have is in regard to this endotoxin. You injected it 48 hours before the infectious challenge and within this period of time, as I recall, in some cases there should be an increase in non-specific resistance capable of protecting them against bacterial challenge.

MARCUS: We chose that time because it has been subscribed to, and we had the same results. There is a very critical time for inducing protection. You remember that this time is quite critical, because if you challenge about four hours after endotoxin administration there is a decrease in resistance. Also, if you wait much beyond three days, it is all gone.

BERRY: Has an increase in non-specific resistance ever been shown to protect against a viral challenge under any condition?

MARCUS: Not that I know of.<sup>6</sup>

BERRY: I don't remember either.

TRAPANI: Does anyone ever use AKR mice for long term experiments on cold exposure? In regard to some of the remarks you made about the neoplasms and virus studies, it would be interesting to study the AKR mouse. This strain of mouse shows spontaneous development of leukemia and lymphoma at about nine months to one year of age, and this condition has been considered to have a viral etiology. It would be interesting to use the AKR

<sup>6</sup> See article by Nemes and Hilleman, Proc. Soc. Exp. Biol. Med. 110: 500, 1962, in which endotoxin is shown to increase resistance in mice against some but not all virus infections.

## SPECIFIC AND NONSPECIFIC RESISTANCE

mouse; just put them into the cold and see what happens.

METCALF: I would like to make just one comment. I think we are all aware of the importance of the genetics of the host that we are using, but I wonder if there might not be more subtle relationships that we may be missing. For example, Weir<sup>7</sup> and others have utilized sub-lines within a given species which vary significantly with regard to blood pH. This may be of importance in phagocytosis and perhaps some of the mechanisms that you are measuring. I wonder if we may be overlooking these relationships and consequently failing to assess or evaluate properly some of the results obtained.

SULKIN: My ears perked up at the comment you made, and I happened to think of the experiments that we recorded a few years ago on bacteriophage clearance in people and animals -- rabbits, in this case. As you know, when bacteriophage has been introduced intravenously into a rabbit it is inactivated quite promptly and you can't detect phage in an animal, actually, within a couple of hours. And we have the impression that perhaps properdin was the component that was inactivated and undertook an experiment in which we treated these animals with zymosan, and zymosan did something that depressed properdin, because phage would then persist, and this has a bearing on the point that Dr. Berry mentioned.

MITCHELL: My one question is this: Dr. Berry and his group and the group at Wisconsin, got to comparing notes. We have experiments wherein one is performed at pressures of about 100 mm less atmosphere than are the ones that are performed at Bryn Mawr or at Wisconsin. I wonder if this small difference may make them into different animals. The reason I am saying this is because I knew of your own experience with altitude exposures, and I don't know whether you combined this with cold.

BERRY: I would like to say that Joe Wilson and I have been

<sup>7</sup> Weir, J. A. 1949. J. Infect. Dis. 84: 252-274.



exchanging mice between Bryn Mawr and Madison because we have not been able to get duplicate results, and they're interesting to both of us. As a matter of fact, when we ship these mice by air express, the mice have been changed. The Madison mice shipped to Bryn Mawr are very different animals.

MITCHELL: Well, it will be interesting when you gentlemen eliminate this additional factor of 100 mm of mercury and see what happens.

# VIRULENCE AS A FACTOR IN HOST RESPONSE TO BACTERIAL INFECTION AT LOW ENVIRONMENTAL TEMPERATURE<sup>1,2</sup>

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## ABSTRACT

The purpose of this report has been to investigate the effect of acute exposure to cold on the response of mice to Salmonella typhimurium and Staphylococcus aureus, and to injections of lipopolysaccharides derived from Gram negative organisms. Mice maintained in individual compartments without bedding following infection with an avirulent strain of either Salmonella typhimurium or Staphylococcus aureus are more susceptible when exposed continuously to 5° C than they are when exposed to 15° C or to 25° C. These differences are not observed when virulent strains are used, while acclimatization to cold for two weeks fails to alter the response to the avirulent organisms. Mice kept at 5° C post-injection are sensitized 250-fold to pasteurized Salmonella typhimurium, and about 10-fold to lipopolysaccharide derived from Serratia marcescens compared to control animals housed at 25° C. Mice given an LD<sub>75</sub> dose of lipopolysaccharide and placed at 5° C for 12 hours before transfer to 25° C are as susceptible to the endotoxin as mice kept continuously in the cold. Conversely, mice given the same dose and retained at 25° C for 6 or 12 hours before placing them at 5° C are almost as resistant as mice kept continuously at 25° C. The period of sensitization to lipopolysaccharide following cold exposure was paralleled by the time at which a drop in body temperature occurred following the low temperature stress and/or endotoxin poisoning. Protection was afforded the cold exposed mice against endotoxin poisoning by exogenously administered cortisone acetate while 8 units of ACTH enhanced the lethal effects of the toxin. The adrenal response of the host to temperature stress seems to be of paramount significance in determining sensitization to lipopolysaccharide.

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Many individuals assume that low environmental temperature plays a role in predisposition to infectious disease. While there is a relative dearth of scientific evidence to support this concept, a few reports have appeared in the literature. Some indicate that cold increases susceptibility to specific infections while others demonstrate an increase in survival following low temperature exposure (Girone, 1962). It seemed of import, therefore, to shed further light on this complex problem. The results described below demonstrate the response of cold exposed mice to infection with Salmonella typhimurium, Staphylococcus aureus, and to injection of endotoxins derived from Gram negative bacteria.

## MATERIALS AND METHODS

During the experimental period,  $21 \pm 2$  gm Carworth farm, CF-1 female mice were housed singly and maintained in plexiglass compartments without bedding. Mice could thus be isolated one from one another so that a group of ten animals could be housed individually in a total area measuring 8 in. x 10 1/2 in. The importance of single housing (Kulka, 1961) and lack of nesting material (Barnett et al., 1959) as stress factors in cold studies have been demonstrated. Single housing eliminates the huddling of mice and conservation of body heat that normally accompanies this activity. Lack of nesting material prevents burrowing and thereby deprives the animal of insulatory material available under usual caging conditions, thus accentuating heat loss. The cold exposed animals were maintained in walk-in refrigerators at  $5 \pm 1^{\circ}$  C or  $15 \pm 1^{\circ}$  C. Room temperature controls were kept in an air-conditioned laboratory at  $25 \pm 2^{\circ}$  C. The animals were given food (Dietrich and Gambrill's pathogen-free mouse biscuits) and water ad libitum. For the single experiment involving high temperature exposure, the mice were placed in compartments held in an incubator with a controlled temperature of  $35 \pm 2^{\circ}$  C. Three liters of air pre-warmed in a water bath were passed through the incubator each minute. The rate of air flow was determined by a flow meter (Fisher).

## VIRULENCE AND BACTERIAL INFECTION

Injections of bacteria derived from 17 hour brain-heart infusion cultures, and lipopolysaccharide derived from Serratia marcescens (Difco), as well as heat-killed Salmonella typhimurium, were suspended in 0.5 ml of non-pyrogenic saline (Baxter's) and administered at the start of each experiment. All injections were given intraperitoneally other than staphylococci which were given intravenously via the tail vein of the mouse. A viable count of  $10^9$  cells per ml based on numerous experimental determinations was assumed for the undiluted cultures. Staphylococcal toxins were obtained as sterile filtrates of the contents of diffusion chambers which were constructed with Viscosedialyzing membranes. Staphylococcus aureus, Giorgio, had been grown inside the chambers for 10 to 14 days after implantation in the peritoneal cavities of mice. This technique is described in detail by Houser and Berry (1961). Mice were injected intravenously with 0.1 ml of a 1:32 dilution of the filtrate and placed immediately at 5° C or 25° C. The material was supplied by Mr. Enoch D. Houser.

Rectal temperatures were determined by inserting a thermistor probe approximately 21 mm into the rectum of mice for twenty seconds. The temperature was read from a telethermometer (Yellow Springs Instrument Company, Yellow Springs, Ohio) to which the probe was connected. A mercury thermometer was used to calibrate the instrument.

Five mg of cortisone was administered subcutaneously as a suspension of cortisone acetate (Nutritional Biochemicals, Cleveland) in 0.5 ml of non-pyrogenic isotonic saline solution. The suspension, stabilized with a drop of Tween 80 detergent was prepared in a glass homogenizer with teflon pestle and used immediately thereafter. Adrenocorticotrophic hormone, ACTH, (Armour Laboratories, Chicago) was injected as a gelatin suspension (Acthar Gel) containing two units per 0.05 ml.

The significance of differences in survival due to experimental treatments was determined by the chi-square test using Yate's corrected formula (Croxtan, 1959) and that for rectal temperature measurements was determined by the rank order test (White, 1952). For some treatments mean survival times were calculated, including only the mice that died. LD<sub>50</sub> dosages were determined



## PREVITE AND BERRY

| Days Post<br>Infection | Number of survivors at |       |       |
|------------------------|------------------------|-------|-------|
|                        | 5° C                   | 15° C | 25° C |
| 0                      | 42                     | 32    | 41    |
| 2                      | 39                     | 32    | 41    |
| 4                      | 32                     | 30    | 40    |
| 6                      | 19                     | 26    | 36    |
| 8                      | 9                      | 20    | 27    |
| 10                     | 3                      | 17    | 22    |
| 12                     | 1                      | 8     | 19    |
| 14                     | 0                      | 8     | 16    |

Table I. Survival of mice infected with  $10^5$  Salmonella typhimurium, SR-11-A. (Derived from data presented in J. Infect. Dis. 110: 201-209. 1962.)

according to the method of Reed and Muench (1938).

## RESULTS

Infection with S. Typhimurium, SR-11-A

Mice were infected intraperitoneally with  $10^5$  viable cells of S. typhimurium, SR-11-A. This strain was derived by chance from an agar slant culture of SR-11 which had been stored at 5° C for a period of several months. On transfer to brain-heart infusion broth and injection into mice, it was found to have lost much of its virulence. The animals were divided into three groups and placed at temperatures of 5° C, 15° C, and 25° C immediately after infection. The pooled results are presented in Table I. None of 42 mice survived at 5° C while 25 per cent (8 of 32) of those at 15° C and 39 per

## VIRULENCE AND BACTERIAL INFECTION

| Days Post Infection | Number of Survivors at |       |       |
|---------------------|------------------------|-------|-------|
|                     | 5° C                   | 15° C | 25° C |
| 0                   | 32                     | 32    | 31    |
| 2                   | 31                     | 32    | 30    |
| 4                   | 25                     | 24    | 20    |
| 6                   | 5                      | 4     | 6     |
| 8                   | 1                      | 0     | 3     |
| 10                  | 0                      | 0     | 3     |
| 12                  | 0                      | 0     | 3     |
| 14                  | 0                      | 0     | 3     |

Table II. Survival of mice infected with  $10^5$  Salmonella typhimurium, SR-11. (Derived from data presented in J. Infect. Dis. 110: 201-209, 1962)

cent (16 or 41) at 25° C were alive at 14 days. Deaths resulted sooner at 5° C than at the two higher temperatures ( $P < 0.005$  in both cases) and mean survival times in animals succumbing to infection were 6.0, 8.5, and 8.7 days in order of increasing temperature. The experiments were terminated at 14 days in order to avoid ambiguities in interpreting the results. Five degree exposure alone can cause deaths if mice of the strain used are maintained at this temperature for periods longer than two weeks.

In a single experiment, 12 mice that had survived acclimatization for two weeks at 5° C were then infected with  $10^5$  SR-11A. Fourteen days later only one of twelve was still alive. Thus, survival at 5° C in mice with this infection does not differ with or without two weeks of prior acclimatization ( $P = 0.3$ ).

### Infection with S. Typhimurium, SR-11

Mice were infected intraperitoneally with  $10^5$  cells of virulent

## PREVITE AND BERRY

| Days Post<br>Infection | 5 x 10 <sup>6</sup> cells |     |     | Infectious Dose<br>5 x 10 <sup>4</sup> cells |     |     | 2 x 10 <sup>4</sup> cells |     |     |
|------------------------|---------------------------|-----|-----|--|-----|-----|---------------------------|-----|-----|
|                        | 5°                        | 15° | 25° | 5°   | 15° | 25° | 5°                        | 15° | 25° |
| 0                      | 10                        | 10  | 10  | 12   | 10  | 10  | 33                        | 10  | 32  |
| 2                      | 6                         | 6   | 4   | 11   | 10  | 10  | 33                        | 9   | 32  |
| 4                      | 0                         | 0   | 0   | 11   | 10  | 10  | 32                        | 9   | 31  |
| 6                      |                           |     |     | 1  | 4   | 6   | 19                        | 3   | 22  |
| 7                      |                           |     |     | 1  | 3   | 5   | 6                         | 2   | 16  |
| 8                      |                           |     |     | 0  | 1   | 3   | 3                         | 2   | 15  |
| 10                     |                           |     |     |  | 0   | 1   | 1                         | 0   | 6   |
| 12                     |                           |     |     |  |     |     |                           |     | 5   |
| 14                     |                           |     |     |  |     |     |                           |     | 4   |

Table III. Survival of mice infected with *Salmonella typhimurium*, SR-11, and exposed to 5° C, 15° C, and 25° C. (Derived from data presented in J. Infect. Dis. 110: 201-209. 1962.)

*S. typhimurium*, strain SR-11. The animals were divided into three groups and placed without delay at 5° C, 15° C, and 25° C. The results in Table II demonstrate no significant difference in survivorship and the mean survival times were, in order of increasing temperature, 5.4, 5.3, and 4.7 days. Cold was thus without influence either on survivorship or survival time of mice infected with this particular dose and strain of *Salmonella*.

The effect of graded doses of SR-11 was then evaluated in order to determine whether an effect of temperature on the infectious process may have been masked by an overwhelming infection. Mice were infected with seven different doses of bacteria, ranging from 500 to 5 million cells and then placed at 5° C, 15° C, and 25° C. The animals were more successful, temporarily, in resisting the lethal effects of the virulent salmonellae when housed at room temperature (Table III). At seven days after infection with 20,000 SR-11 only 18.2 per cent (6 of 33) of the mice survived at 5° C, whereas 50 per

## VIRULENCE AND BACTERIAL INFECTION

| Dats Post<br>Infection | Number of Survivors at |       |       |
|------------------------|------------------------|-------|-------|
|                        | 5° C                   | 15° C | 25° C |
| 0                      | 22                     | 22    | 24    |
| 2                      | 15                     | 22    | 22    |
| 4                      | 12                     | 18    | 21    |
| 6                      | 11                     | 14    | 20    |
| 8                      | 10                     | 11    | 19    |
| 10                     | 10                     | 10    | 19    |
| 12                     | 2                      | 8     | 19    |
| 14                     | 2                      | 8     | 19    |

Table IV. Survival of mice infected with  $10^8$  *Staphylococcus aureus*.  
(Derived from data presented in J. Infect. Dis. 110: 201-209, 1962.)

cent (16 of 32) survived at 25° C ( $P < 0.02$ ). This difference was abolished by the fourteenth day. With infectious doses as low as 500 cells or as high as 5 million cells, both survival time and survivorship of mice infected with SR-11 were largely independent of the environmental temperature at which the post-infection period was spent.

### Infection with *Staphylococcus*

Mice were infected intravenously with  $10^8$  cells of a relatively avirulent variant culture of *Staphylococcus aureus*, Giorgio. They were placed immediately after infection at 5° C, 15° C, or 25° C. After 14 days, 9 per cent (2 of 22) survived at 5° C, 36 per cent (8 of 22) survived at 15° C, and 79 per cent (19 of 24) survived at 25° C. Thus with this particular organism, survival was decreased significantly by 5° C ( $P = 0.001$ ) or 15° C exposure ( $P = 0.01$ ) when compared to that at 25° C (Table IV).

Mice injected intravenously with  $10^8$  virulent *Staphylococci* were about equally susceptible both as to survivorship and survival time



5° C and 25° C. Three often animals survived at each temperature, and the mean survival times were 2.9 days at 5° C and 3.6 days at 25° C.

With a smaller infectious dose of virulent cells (500 bacteria), four of eight mice survived at 5° C while six of eight were alive at 25° C within 14 days of the initial infection ( $P > 0.05$ ). Once again as with salmonellosis, the more virulent organism seems to produce a fatal infection with little influence from the environmental temperatures at which the animals were housed.

#### Injection of Staphylococcal Toxin

A total of 16 mice were injected intravenously with 0.1 ml of a 1:32 dilution of a staphylococcal toxin derived as a sterile filtrate from a viscose diffusion chamber in which the bacteria had been grown as described previously. Ten were placed at 5° C and the remainder were held at 25° C. Three died at 5° C and none at 25° C. For the number of animals involved this is not significant. However, if all mice previously given this amount of toxin (by Mr. E. D. Houser) at room temperature are added to the 25° C group (a total in excess of 30, none of which died), it would suggest that cold sensitizes mice to Staphylococcal toxins. The limited supply of toxin prevented a more extensive test.

#### Heat-killed *S. Typhimurium*

Groups of 10 mice were given graded numbers of pasteurized *S. typhimurium* intraperitoneally. The LD<sub>50</sub> dose at 5° C was  $8 \times 10^6$  cells while at 25° C, it was  $2 \times 10^9$  cells. Thus, cold exposure increases 250-fold the lethal effects of heat-killed salmonellae.

Two groups of mice were then acclimatized to 5° C and 25° C for 28 days. At the end of this period,  $1.6 \times 10^8$  heat-killed cells were injected, and both groups of mice were placed at 5° C. Nine of ten cold acclimatized mice survived, while only two of ten non-cold acclimatized animals lived ( $P = 0.008$ ). However, 20 per cent or more of the original group of mice at 5° C usually die during a

# VIRULENCE AND BACTERIAL INFECTION

| Days Post<br>Injection | Controls<br>5° C | Treatment and Survivors |                 |                 |
|------------------------|------------------|-------------------------|-----------------|-----------------|
|                        |                  | 25° C                   | 12 Hrs.<br>5° C | 21 Hrs.<br>5° C |
| 0                      | 40               | 10                      | 30              | 10              |
| 1                      | 9                | 10                      | 14              | 0               |
| 2                      | 9                | 10                      | 14              | 0               |
| Per cent<br>Survival   | 22.5             | 100.0                   | 46.7            | 0               |

Table V a. Sensitization of Lipopolysaccharide in the cold. All mice received 60-65  $\mu$ g of Endotoxin. (Derived from data presented in J. Infect. Dis. 110: 201-209. 1962.)

| Days Post<br>Injection | Controls<br>5° C | Treatment and Survivors |                 |                  |
|------------------------|------------------|-------------------------|-----------------|------------------|
|                        |                  | 25° C                   | 6 Hrs.<br>25° C | 12 Hrs.<br>25° C |
| 0                      | 40               | 10                      | 30              | 15               |
| 1                      | 9                | 10                      | 22              | 15               |
| 2                      | 9                | 10                      | 21              | 15               |
| Per cent<br>Survival   | 22.5             | 100.0                   | 70.0            | 100.0            |

Table V b. Protection against Lipopolysaccharide by 25° C exposure. All mice received 60-65  $\mu$ g of Endotoxin. (Derived from data presented in J. Infect. Dis. 110: 201-209. 1962.)

month long acclimatization. Therefore, it is not completely clear whether acclimatization to cold actually enhances the resistance of the mice to endotoxin or merely serves to select more resistant animals for the study.

#### Injection of *Serratia Marcescens* Lipopolysaccharide

The LD<sub>50</sub> doses of *S. marcescens* lipopolysaccharide (Difco) were determined to be 38.6  $\mu$ g, 408  $\mu$ g, and 162.5  $\mu$ g at ambient temperatures of 5° C, 25° C, and 35° C respectively. Therefore, both low and high environmental temperature sensitize mice to lipopolysaccharide.

#### Timing the Effects of Endotoxin at Different Temperatures

Mice were given 60-65  $\mu$ g of endotoxin (*S. marcescens* lipopolysaccharide) placed immediately at 5° C, and at intervals thereafter returned to room temperature. Exposure to cold for 12 hours (46.7 per cent survival) sensitizes mice to bacterial lipopolysaccharide almost as completely as continuous exposure (22.5 per cent survival) ( $P > 0.05$ , Table Va). Survival of 25° C controls (100 per cent) is significantly different from that of 5° C controls (22.5 per cent,  $P < 0.001$ ). It is evident that the response of cold exposed mice to this dose of endotoxin is determined to a great extent within a 12 hour period. Experiments with heat-killed salmonellae ( $1.6 \times 10^8$  cells, which is more than an LD<sub>50</sub> dose), or with smaller doses of lipopolysaccharide (40  $\mu$ g) produced similar results.

Mice were then given 60-65  $\mu$ g of lipopolysaccharide, retained at room temperature, and at intervals thereafter placed in the 5° C room. Survivorship in the groups maintained at 25° C for six hours (70 per cent) and twelve hours (100 per cent) differed significantly from that of mice continuously exposed to 5° C after endotoxin injection (22.5 per cent,  $P < 0.001$  in both cases; Table Vb). With a lower dose of lipopolysaccharide (40  $\mu$ g) similar results were obtained. Thus the mouse at normal room temperature (25° C) is able to render non-lethal within a 6-12 hour period a dose of endotoxin that is about 75 per cent lethal at an ambient temperature of 5° C.

# VIRULENCE AND BACTERIAL INFECTION

| Time Post<br>Injection | Treatment and Temperatures |                    |  |
|------------------------|----------------------------|--------------------|--|
|                        | 25° C Controls             | 5° C Controls      | 25° C & 408ug LPS<br>5° C & 50ug LPS     |
| 0 Hrs.<br>(9:15 a.m.)  | 37.4 ± 0.4<br>(10)         |                    |  |
| 6 Hrs.                 | 37.5 ± 0.5<br>(10)         | 36.1 ± 0.5<br>(10) | 36.4 ± 0.9<br>(10)<br>29.6 ± 3.5<br>(20) |
| 12 Hrs.                | 37.9 ± 0.6<br>(10)         | 36.3 ± 0.5<br>(10) | 34.8 ± 1.7<br>(10)<br>29.7 ± 4.6*<br>(9) |
| 24 Hrs.                | 36.6 ± 0.8<br>(10)         | 36.2 ± 0.4<br>(10) |  |

Table VI. Average rectal temperatures of Lipopolysaccharide poisoned mice. \*The rectal temperature of moribund mice was frequently below the lower limits of the telethermometer (20° C). In these cases the temperature was recorded as 20° C in order to compute the arithmetic mean for the number of animals listed in parentheses. Each value represents the mean plus or minus the standard deviation from the mean of the number of animals studied.



Rectal Temperatures

Endotoxin has been reported to elicit hypothermia in mice (Halberg and Spink, 1956; Berry et al., 1959). It seemed appropriate to determine whether or not the onset and duration of hypothermia coincided with the period during which the mice were sensitized to this poison by cold exposure. The rectal temperatures of mice maintained at 25° C versus those at 5° C differed significantly ( $P < 0.001$ ) after six (37.5° C vs. 36.1° C respectively) and 12 hours of exposure (37.9° C vs. 36.3° C). These differences disappeared within 24 hours at the two different ambient temperatures ( $P > 0.05$ ) (Table VI).

The rectal temperature of 25° C controls (37.5° C) was significantly different ( $P < 0.05$ ) from that recorded in mice that had received an LD<sub>50</sub> dose of lipopolysaccharide six hours previously (36.4° C). Within twelve hours the difference was even more marked between these two groups (37.9° C vs. 34.8° C respectively,  $P < 0.001$ ). The rectal temperature of 5° C control mice and that of mice given an LD<sub>50</sub> dose of lipopolysaccharide prior to exposure to this same temperature differed markedly at 6 hours (36.1° C vs. 29.6° C) and at 12 hours (36.3° C vs. 29.7° C,  $P < 0.001$  in both cases).

Thus hypothermia is evident within 6-12 hours in 5° C controls as well as lipopolysaccharide-poisoned animals exposed to 25° C. It is most severe in lipopolysaccharide-poisoned animals maintained at 5° C post-injection.

Hormone Administration and Survivorship

Cortisone acetate. It has been reported that adrenalectomized rats are killed by one-thousandth the dose of endotoxin required to kill normal animals (Brooke et al., 1959). The report that the administration of exogenous corticoids protects endotoxin poisoned animals was described as early as 1951 by Duffy and Morgan and has been repeatedly confirmed since then (Berry et al., 1959). However, whether the same protective effect would be evident in animals whose metabolism was elevated by cold exposure remained to be determined.

## VIRULENCE AND BACTERIAL INFECTION

| Days Post<br>Injection | 50ug<br>LPS | Corti-<br>sone &<br>50ug LPS | 70ug<br>LPS | Corti-<br>sone &<br>70ug LPS | Cortisone<br>Controls |
|------------------------|-------------|------------------------------|-------------|------------------------------|-----------------------|
| 0                      | 30          | 26                           | 40          | 30                           | 10                    |
| 1                      | 19          | 23                           | 0           | 24                           | 10                    |
| 2                      | 19          | 23                           | 0           | 24                           | 10                    |
| Per cent<br>Survival   | 63.3        | 88.5                         | 0           | 80.0                         | 100.0                 |

Table VII. Hormonal protection against Lipopolysaccharide. Treatment and survivors.

With this in mind, five mg of cortisone acetate was administered subcutaneously immediately before intraperitoneal injection of 50  $\mu$ g of lipopolysaccharide (endotoxin). When the animals were maintained at 5° C post-injection survivorship was slightly but not significantly greater ( $P > 0.05$ ) in hormone treated mice than in animals not receiving hormone (88.5 per cent vs. 63.3 per cent, Table VII). However, with a larger quantity of lipopolysaccharide, the protection afforded by cortisone was clearly demonstrated. When an LD<sub>100</sub> dose (70  $\mu$ g) of lipopolysaccharide was given to mice exposed to 5° C after injection, all 40 controls died, while 24 of 30 mice given 5 mg of cortisone acetate survived ( $P < 0.001$ ). Cortisone alone killed no animals.

### Adrenocorticotrophic Hormone

Exogenous ACTH administration has been reported to sensitize mice to the lethal effects of endotoxin. This occurs presumably because glycocorticoids are released too promptly in animals that receive both endotoxin and ACTH (Berry and Smythe, 1959; 1961) to fulfill the protective action the corticoids normally afford against endotoxin poisoning alone. The experiments described below were carried out to determine whether or not cold exposed animals would be further sensitized to lipopolysaccharide if given ACTH. The re-

## PREVITE AND BERRY

| Days Post Infection | 50ug LPS | 2 Units ACTH & 50ug LPS | 8 Units ACTH & 50ug LPS | 8 Units ACTH |
|---------------------|----------|-------------------------|-------------------------|--------------|
| 0                   | 30       | 30                      | 30                      | 14           |
| 1                   | 19       | 15                      | 3                       | 14           |
| 2                   | 19       | 15                      | 3                       | 14           |
| Per cent Survival   | 63.3     | 50.0                    | 10.0                    | 100.0        |

Table VIII. Sensitization to Lipopolysaccharide by ACTH. Treatment and survivors.

sults indicate that this does occur (Table VIII). Two or eight units of ACTH were administered subcutaneously immediately before 50  $\mu$ g of lipopolysaccharide and subsequent exposure to 5° C. Survival in controls (63.3 per cent) was slightly but not significantly higher ( $P>0.05$ ) than that of animals receiving 2 units of ACTH (50 per cent), but was significantly higher ( $P<0.001$ ) than that in animals receiving 8 units of this hormone (10 per cent survival). Eight units of ACTH alone did not kill any of 14 mice.

## DISCUSSION

Following experimental infection with *S. typhimurium*, the greater susceptibility of cold exposed mice (5° C, measured in terms of both survivorship and survival time) compared to those maintained at usual room temperature (25° C) is evident only when a comparatively avirulent strain is used (SR-11-A). Thus, a situation in which the outcome of a host-parasite interaction is intimately linked to the genetic constitution of the microorganism involved is evident. This may be related to the findings of Schneider (1949) and Schneider and Zinder (1956). They showed that diet altered the out-

## VIRULENCE AND BACTERIAL INFECTION

come of salmonellosis only when a mixed population of virulent and avirulent pathogens was inoculated into the mice. As an alternative possible explanation, the results of Muschenheim et al., (1943) should be mentioned. They found that in rabbits infected with a virulent Type I strain of pneumococcus the only effect of reduced body temperature was a decrease in the local inflammatory response. However, with a relatively avirulent Type III strain, overwhelming bacteremia and death resulted as well. These investigators concluded that reduction in body temperature can alter resistance to infection with an otherwise relatively avirulent pathogen. Despite differences in experimental approach, the results reported in this paper tend to support such a concept.

If, as is reported by Berry and Smythe (1960), endotoxins contribute to the toxic manifestations of salmonellosis, then the close similarity of survivorship and survival time in animals infected with virulent SR-11, regardless of subsequent temperature exposure, might be the result of the formation of a smaller amount of endotoxin at 5° C than at 25° C. This argument would follow, since cold exposed mice are killed with less endotoxin than those maintained at normal temperatures. In line with this hypothesis, animals under optimal environmental conditions might support more rapid growth of certain virulent pathogens than animals in the cold. Perhaps infection with the avirulent SR-11-A results in more deaths in a shorter time at 5° C than at 25° C because the lethal level of endotoxin accumulates sooner at the former temperature. The defenses of the mouse might be sufficiently impaired by cold to permit a steady in vivo proliferation of the bacteria which ultimately leads to death, in contrast with the events in animals under more normal environmental temperatures.

The results with Staphylococcus aureus infections seem to fit into the interpretations suggested above. The relationship between virulent and avirulent S. aureus parallels closely the findings with salmonellosis. Additional studies will be required, however, before the role of cold in active infections can be clearly interpreted.

From our results it appears that the host is able to overcome certain effects of endotoxin within the first 6 to 12 hours of administration. Reports available in the literature suggest too that host



response to endotoxin is prompt and dramatic (Conti et al., 1961; Berry and Smythe, 1961). The period of sensitization to lipopolysaccharide by cold exposure (Tables Va and Vb) is paralleled by the time at which a drop in body temperature occurs following cold exposure and/or endotoxin poisoning (Table VI). Whether the reduction in rectal temperatures is the cause or result or merely reflects metabolic changes that account for sensitization remains unanswered. However, the latter view seems more likely in view of recently obtained but yet unpublished data from this laboratory.

Sensitization of mice to bacterial endotoxin by cold exposure may be related to adrenocortical function. If the stress of cold results in an initial hyperactivity of the gland, an alarm reaction would occur as part of a general adaptation syndrome (Selye, 1955). Adrenal activity is said to level off after an initial rise following exposure to cold (Heroux and Hart, 1954a; Schonbaum, 1960), while the low cholesterol content of the adrenals of room temperature exposed mice 17 hours after endotoxin administration has been interpreted as being the result of an earlier hyperactivity of these glands (Berry and Smythe, 1961). In view of the protection cortical hormones afford against endotoxin (Brooke et al., 1959), cold stress (Heroux and Hart, 1954b), or both of these factors combined (Table VII), the following postulate does not seem unreasonable. The greater resistance to endotoxin of cold-acclimatized mice compared to non-acclimatized animals may involve a greater capacity of the adrenal of the former to release protective corticoids at the proper time. In rats exposed to 5° C, the maximal activity of the adrenal cortex as shown by P<sup>32</sup> uptake occurs at 2 hours after exposure (Rossiter and Nicholls, 1957). Thus it may be that an initial depletion of corticoid reserves occurs in the cold exposed mouse prior to the time at which these hormones are needed for protection against endotoxin. "Functional adrenalectomy" due to temperature stress, therefore, may be responsible for sensitization of cold exposed mice to endotoxin, just as surgical adrenalectomy has been reported to do so in rats (Brooke et al., 1959).

While the present report emphasizes adrenal cortical involvement in protection or sensitization to endotoxin following cold exposure, it is our opinion that more data must be gathered concerning other facets of endocrine involvement, metabolism, and host defense

## VIRULENCE AND BACTERIAL INFECTION

mechanisms before definitive and final statements can be made concerning this problem.

### SUMMARY

Mice maintained in individual compartments without bedding following infection with an avirulent strain of either Salmonella typhimurium or Staphylococcus aureus are more susceptible when exposed continuously to 5° C than they are when exposed to 15° C or to 25° C. These differences are not observed when virulent strains are used, while acclimatization to cold for two weeks fails to alter the response to the avirulent organisms. Mice kept at 5° C after injection are sensitized 250-fold to pasteurized S. typhimurium and about 10-fold to lipopolysaccharide derived from Serratia marcescens compared to control animals housed at 25° C. Mice given an LD<sub>75</sub> dose of lipopolysaccharide and placed at 5° C for 12 hours before transfer to 25° C are as susceptible to the endotoxin as mice kept continuously in the cold. Conversely, mice given the same dose and retained at 25° C for 6 or 12 hours before placing them at 5° C are almost as resistant as mice kept continuously at 25° C. The period of sensitization to lipopolysaccharide following cold exposure was paralleled by the time at which a drop in body temperature occurred following the low temperature stress and/or endotoxin poisoning.

Protection was afforded the cold exposed mice against endotoxin poisoning by exogenously administered cortisone acetate, while 8 units of ACTH enhanced the lethal effects of the toxin. The adrenal response of the host to temperature stress seems to be of paramount significance in determining the sensitization to lipopolysaccharide.

PREVITE AND BERRY

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## DISCUSSION

BLAIR: I was particularly interested in the development of the hypothermia of the *Serratia* endotoxin. I wonder if here again we aren't faced with the problem of the species differences in experimental work of this nature. Maxwell, several years ago, using dogs -- I am sorry, you used *Serratia* endotoxin also -- uniformly obtained hyperpyrexia, and his animals died in the classic picture of septic shock with a high fever.

PREVITE: In response to that, I would say that mice seem to be peculiar animals in that respect. Although I don't profess to have tremendous knowledge concerning the effects of endotoxin, from what I have read, it seems as if the mouse is one of the few animals that responds to this poison with a lowered body temperature. Most animals, including man and rabbits, respond with hyperthermia. These divergent responses again point out significant species differences.

## VIRULENCE AND BACTERIAL INFECTION

BLAIR: I ask what the condition of the mice was at the 5° C exposure. That is, after development of hypothermia, whether they were lethargic or comatose or not just before they died?

PREVITE: You are referring to the control mice placed at 5° C?

BLAIR: No, the ones that were infected.

PREVITE: Measurements weren't made following infection; they were made following endotoxin injection. But these animals, after 6 to 12 hours with and LD<sub>50</sub> dose, were pretty sick. They did not move in the cage. Within eight hours, one could practically predict by its rectal temperature that endotoxin-poisoned, cold stressed mouse which was going to die or survive. Animals whose rectal temperatures dropped to as low as 29.5° C always died. And those animals whose temperatures were 33° C to 34° C or higher usually survived what was potentially a lethal dose of endotoxin.

SCHMIDT: But in your third to the last slide, I think you were showing us the effects of endotoxins on the temperature of the animal. I may be mistaken, but as I recall, you were indicating for your control animals at 25° C that the rectal temperature was 37.4° C, and then, as the hours progressed, the animals at 5° C showed somewhat lowered rectal temperatures in the range of 36° C, and then you said that at 24 hours, this difference had been overcome.

PREVITE: The difference between the 25° C group and the 5° C group?

SCHMIDT: Yes, but what I think is significant is that the ones at 25° C were now approximately 1° C lower in rectal temperature than they were when they started. In other words, you got a fluctuation of 1° C normally.

PREVITE: If you take the average without considering the deviation, yes, but if you compare these statistically, because of the overlap from one mouse to another, there is no significant

difference after 24 hours. Therefore, my findings would essentially agree with those of Dr. Miya. That is, within 24 hours, the body temperature of control animals exposed to  $5^{\circ}\text{C}$  returns to the normal range.

SCHMIDT: You have essentially shown us that you can take a group of ten animals and leave them at  $25^{\circ}\text{C}$ , and their average rectal temperature is going to drop  $1^{\circ}\text{C}$ .

PREVITE: It is not one complete degree. It's a fraction of a degree. Again, if one compares the figures  $37.8^{\circ}\text{C}$  versus  $36.6^{\circ}\text{C}$  alone, they do seem to differ significantly. However, upon statistical analysis they are not significantly different because of the wide mean deviation and overlap between them.

BERRY: I would like to comment first on Mr. Schmidt's question. It is very relevant. It seems a little peculiar, when you look at the cold figures, to find temperature differences, but it may be just a statistical fluke. Mice show variations in temperature during the day, and particularly, with time of day. This is a well known fact. Around 4:00 a.m., or at least early morning, the temperature is typically a full degree lower than it is in the afternoon. This has been studied extensively by people interested in biological clocks. Now, with any group of mice, in order to establish a change in body temperature with time of day, it is necessary to work with large numbers of animals and to maintain, if possible, a constant environment. With a small sample of mice, it may be possible to get nearly a degree's temperature average difference just because of a sampling anomaly.

WALKER: I think you can get as much of a temperature difference as that and more in addition to that with just a little bit of activity.

BERRY: Yes, and you probably change their temperature with rectal probes.

WALKER: Wisconsin mice don't like a rectal probe at first, and they usually struggle, which will run the body temperature up. If you let them struggle and keep the probe there, you can follow his temperature climb. If you take ten mice in a group

## VIRULENCE AND BACTERIAL INFECTION

and put them into the cage, the tenth mouse will have a temperature one degree higher than the first one just because of your reaching in and stirring these animals up.

PREVITE: Regarding that, I noticed that when a timer with a bell was used in order to leave the rectal thermistor probe in the cold exposed mouse for exactly 15 seconds, the stress of the ringing bell at the end of this period seemed to affect the rectal temperature measurement. Rectal temperatures were lower in mice for which a wristwatch was used to time the 15 second period of thermistor probe insertion. Apparently the sound of the bell served as a stress which could affect body temperature measurements.

BLAIR: Adolph at Rochester and Crisman at Stanford, in order to produce hypothermia in mice and rats, used no anesthesia. It was necessary to restrain the animals. If they were not restrained, no matter how long they were exposed in an environment of  $0^{\circ}\text{C}$  to  $5^{\circ}\text{C}$ , they do not achieve a hypothermia.

WALKER: What is meant by hypothermia? How low is it?

BLAIR: He has taken it down as low as  $18^{\circ}\text{C}$  or  $15^{\circ}\text{C}$ , but the strain on the mice and the initial activity causes elevation in the body temperature, but within several hours they are cooled down quite extensively, as opposed to the group which is allowed to run around in the cages. So the strain is important. This is related to the muscle activity you are talking about.

REINHARD: What was your statement relative to the genetics of the organism and the host-parasite relationship?

PREVITE: We noted that there is a difference between virulent and avirulent strains. The genetic constitution of the parasite can be very important in determining the outcome of the infection as modified by cold exposure.

REINHARD: In a homozygous physiologically standardized host?

PREVITE: I made no reference to the host at all, but I would



agree with you, certainly, the genetic constitution of the host can be just an important.

WALKER: I think we should not expect to see an effect with a very virulent strain or with a virulent virus in a very susceptible host. These are not the circumstances in which you can see small differences. It is very important to have the strain and host under the right circumstances; it seems to me we can then see an effect of temperature. It's ridiculous to expect a measurable effect under all circumstances. Much of the variation in the results reported from experimental work concerned with the effect of cold on infection can be explained, I think, by the variation in host and strain of infectious agent used in the experimental work.

TRAPANI: I am a little curious about something on which you might like to comment. I'm thinking about the effect of ACTH and corticoids, especially. We always think about their possible effect on the host. However, Dr. Miya's results showed that bacteria can change, too, with the animal. Is it possible that there is also an effect on the invading organism? Is there any other work which points to this?

MIRAGLIA: I will comment on this later on this afternoon.

PREVITE: The data I presented would give us little direct information regarding the role of adrenal corticoids and the in vivo infectious process. My work thus far with adrenal corticoids has been centered around the effect of these hormones on the response of mice to heat killed cells or commercially prepared endotoxin.

# ENDOGENEOUS AND EXPERIMENTAL PERITONITIS AND BACTEREMIA IN HYPOTHERMIC MICE

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## ABSTRACT

In experiments with mice, hypothermia at 22° C to 23° C, when maintained for more than 40 hours, almost invariably resulted in the emergence of bacteria in peritoneal fluid and blood. *Klebsiellae*, rapidly eliminated in normal mice, diminished in number only during a period of 2-4 hours, but thereafter increased again in hypothermic mice and caused a profuse bacteraemia. As antibacterial treatment reduced the incidence of peritonitis and bacteraemia without increasing the average survival time in hypothermia, the bacterial invasion is not likely to be of major importance for debilitation and death, but rather a concomitant phenomenon. In subsequent series of experiments, groups of mice were inoculated intraperitoneally with pneumococcal suspensions. The following observations were made; the increase in numbers of pneumococci in the blood was slower in hypothermic mice, the establishment of bacteraemia occurred later in such mice, and pneumonic alterations in the lungs were less common in hypothermic mice. In warming up of hypothermic mice, the events in all these respects were accelerated to equalize the eventual results in these animals with those of the normothermic controls.

## I. Endogenous Peritonitis and Bacteremia

The concept of "endogenous bacteremia" has been created in connection with studies on the effect of irradiation and cortisone treatment of animals (Bennet et al., 1951; Berlin et al., 1952; Fallowfield, 1962; Gledhill and Rees, 1952; Hammond et al., 1954; Miller et al., 1950; Miller et al., 1952; Philipson and Laurell, 1958; Sanders et al., 1957). It is conceivable that this condition, generally appearing as an invasion of the blood stream by bacteria normally present in the intestine, may also take place in hypothermia, as this state is known to interfere with several mechanisms taking part in the anti-

bacterial defense of the body, as antibody formation (Lindner and Tunevall, 1958) leucocytotic reaction (Fedor et al., 1958; Helmsworth et al., 1955; Villalobos et al., 1955), phagocytosis (Fedor et al., 1958) or local tissue reactions (Beyer, 1956; Sanders et al., 1957; Szilagyi et al., 1956). As early as 1897, Fischl observed fatal septicemia in rabbits subjected to chilling.

More recently, the possibility of bacteremia has been studied by Fedor et al. (1956) in dogs maintained at  $28^{\circ}\text{C}$  to  $29^{\circ}\text{C}$  for 6 to 12 hours. In such animals no endogeneous bacteremia occurred, and the ability to clear the blood stream from injected bacteria was very slightly impaired. On the other hand, Billingham (1957) observed in extreme hypothermia an increased passage of bacteria through the endothelial lining of the gut. Having observed at autopsies of mice subjected to prolonged hypothermia the frequent occurrence of peritonitis and bacteremia, we have undertaken some experiments in order to study more closely the development of these conditions and to establish their importance for the mortality of mice kept in the hypothermic state. Further, as a complement to this study, an investigation of the ability of hypothermic mice to eliminate injected bacteria from the blood stream was undertaken.

## METHODS

Inbred albino mice weighing 15 to 20 gm were used for the experiments. The procedure for bringing mice into a controlled hypothermic state and maintaining them there has been described in a previous paper (Lindner and Tunevall, 1958) and will not be repeated here in detail. Briefly, the animals were pre-treated with chlorpromazine-HCl (Hibernal) and ethyl-(1-methyl-butyl)-malonyl-carbamide-Na (Nembutal) before being immersed into a water bath where they were, under administration of oxygen, kept at a body temperature of  $22^{\circ}\text{C}$  (registered rectally).

Intravenous injections were made into the dorsal vein of the tail with a No. 20 "Record" cannula on a syringe of the tuberculin type.

## PERITONITIS AND BACTEREMIA IN MICE

For bacterial counts, blood samples of 0.02 ml were drawn with the same type of syringe prefilled with 0.18 ml of 0.85 per cent NaCl solution after cutting the dorsal vein of the tail thoroughly cleaned with 70 per cent ethyl alcohol. The resulting 1:10 dilution was spread over a 3 per cent horse blood agar plate. After incubation overnight at 37° C, the bacterial growth was roughly estimated as "(+)" (1-5 colonies "+") (6-50), "++" (51-500), or "+++" (more than 500). In the elimination studies, appropriate dilutions of the samples were made to permit a precise count.

Fecal specimens were taken from the rectum with a 2 mm platinum loop and spread on plates as above.

At autopsy, fecal specimens were taken as described above, blood samples from heart puncture were cultured as was described for the tail blood, and peritoneal fluid was collected with a cotton swab from the abdominal cavity and directly spread on a blood agar plate.

## RESULTS

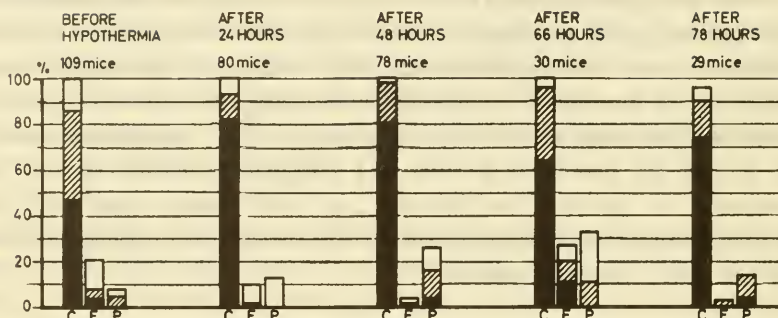
### Influence of Hypothermia on the Intestinal Flora

As the main source of endogeneous bacteremia is likely to be the intestine, the composition and density of the aerobic fecal flora were studied after various periods of hypothermia. The results of fecal cultures are presented in Figure 1a; point of time is represented by numbers of cultures ranging from 109 to 29. Within the coliform group, the proportions of E. coli, A. aerogenes, and Paracolo-  
bacteria were not significantly altered during the experiments. Small numbers of bacteria of other species not represented in the figure were observed but are disregarded here for simplicity. It is evident that the intestinal flora as represented by fecal cultures was not altered significantly in prolonged hypothermia.



## TUNEVALL AND LINDNER

A Not treated



B Treated

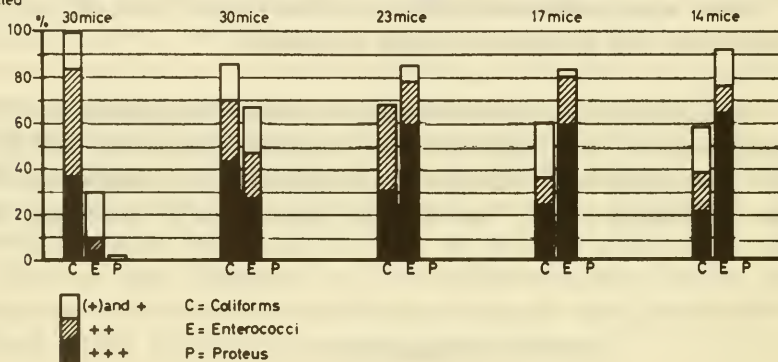


Figure 1. Results of fecal cultures after different periods of hypothermia.

### The Development of Bacteremia in Hypothermic Mice

In four consecutive experiments, in which 34 mice not anti-bacterially treated were used, repeated blood cultures were made after various periods of hypothermia. The results are reported separately for the four experiments in Figure 2. In 8 mice bacteremia occurred after only 24 to 29 hours of hypothermia. After 48 to 54 hours, the number of bacteremic animals had increased to 14. After 78 hours, only three mice remained alive and free from bacteria in their blood, and of these, two mice soon succumbed in a bacteremic state.

The same events are described for all four experiments together in Figure 3, this time with different bacterial species re-

# PERITONITIS AND BACTEREMIA IN MICE

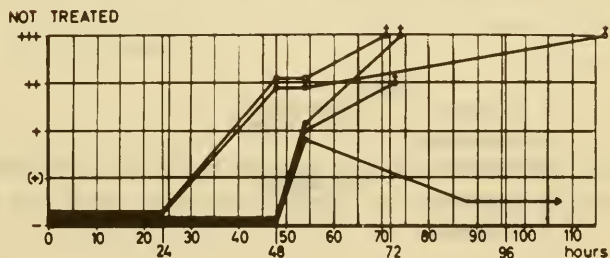
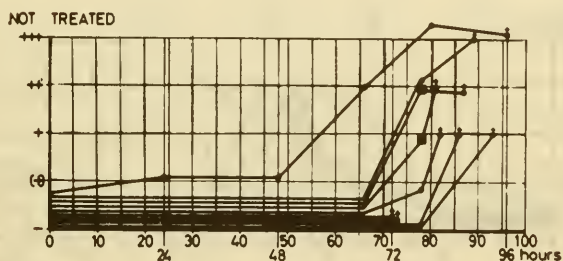
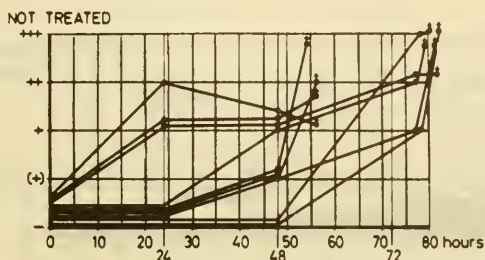
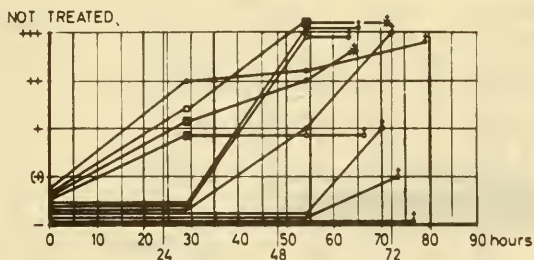


Figure 2. Results of blood cultures made after various periods of hypothermia on 39 mice not antibacterially treated. (+) = 1-5 colonies, + = 6-50, ++ = 51-500, +++ = more than 500.

# TUNEVALL AND LINDNER

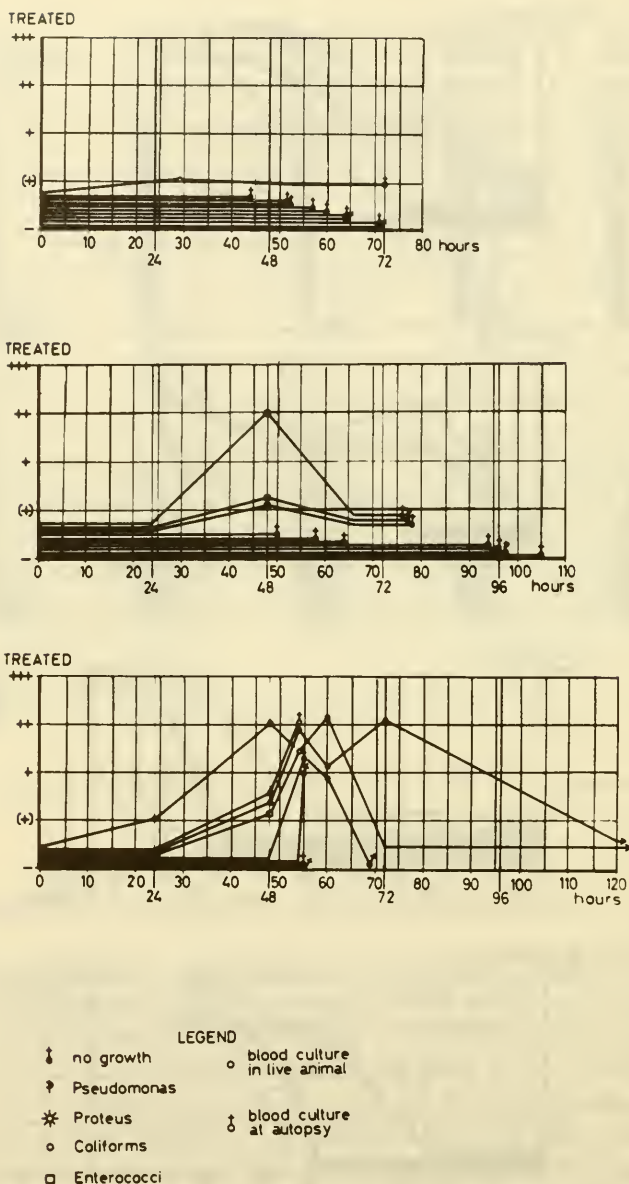


Figure 2 (cont.). Results of blood cultures made after various periods of hypothermia on 28 antibacterially treated mice.

## PERITONITIS AND BACTEREMIA IN MICE

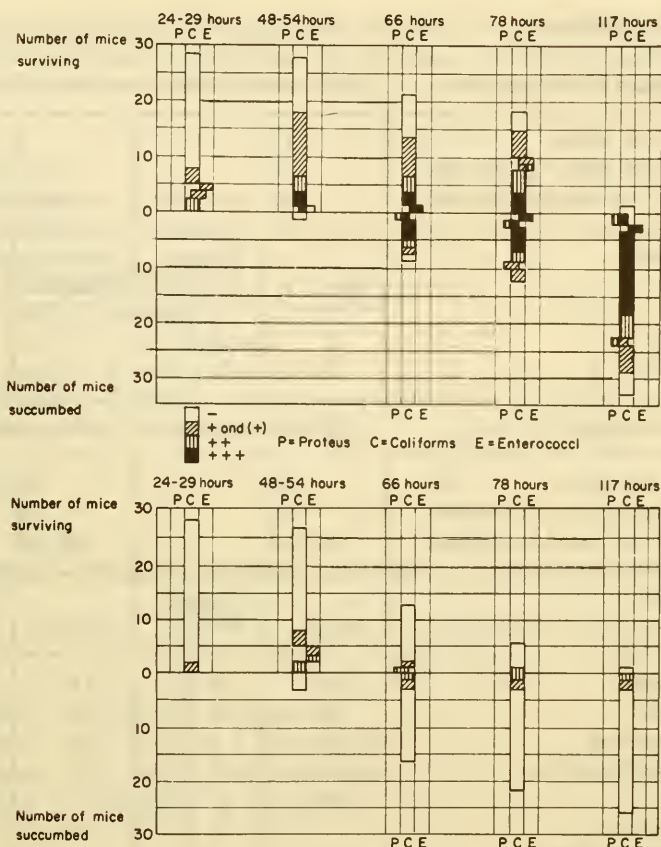


Figure 3. Results of blood cultures in 62 mice, 28 of which were treated antibacterially. The height of the columns above zero line indicates the number of living mice; under this line that of dead mice. Top = 34 mice not treated. Bottom = 28 mice treated with sulpha and streptomycin.

ported separately. In no case was an organism found in the blood which had not been present in feces at the onset of bacteremia.

### Peritonitis and Bacteremia in Sacrificed and Succumbed Mice

Cultures from peritoneal fluid and from blood were made in 30 mice sacrificed in good condition and in 62 mice which had died



# TUNEVALL AND LINDNER

## A. Not treated

Mice sacrificed after                      41 - 48 hours                      49 - 60 hours

| Number of mice          | 17     |       |   | 3      |       |   |
|-------------------------|--------|-------|---|--------|-------|---|
| Mice with pos. cultures | 4      |       |   | 1      |       |   |
| Thereof in perit./blood | 2/3    |       |   | 0/1    |       |   |
| Organism                | Perit. | Blood |   | Perit. | Blood |   |
|                         |        | +++   | + |        | +++   | + |
| E. coli                 | 2      | 2     |   |        | 1     |   |
| Paracoli                | 2      | 1     | 1 |        |       |   |

Mice succumbed after                      41 - 48 hours                      49 - 96 hours

| Number of mice          | 22     |       |   | 40     |       |   |
|-------------------------|--------|-------|---|--------|-------|---|
| Mice with pos. cultures | 18     |       |   | 38     |       |   |
| Thereof in perit./blood | 17/13  |       |   | 36/35  |       |   |
| Organism                | Perit. | Blood |   | Perit. | Blood |   |
|                         |        | +++   | + |        | +++   | + |
| E. coli                 | 12     | 8     | 4 | 31     | 20    | 8 |
| Paracoli                | 1      | 1     |   | 3      | 1     | 1 |
| A. aerogenes            | 2      |       |   | 2      | 2     | 1 |
| Alcalig.faecalis        | 3      | 1     |   | 2      | 1     |   |
| Proteus                 | 3      | 1     | 1 | 4      | 3     | 3 |
| Enterococci             |        | 1     |   | 3      |       |   |
| B. mesentericus         |        |       |   | 1      | 1     |   |

## B. Treated with sulpha + streptomycin

Mice succumbed after                      41 - 48 hours                      49 - 105 hours

| Number of mice          | 1      |       |   | 25     |       |   |
|-------------------------|--------|-------|---|--------|-------|---|
| Mice with pos. cultures | 0      |       |   | 6      |       |   |
| Thereof in perit./blood | 0/0    |       |   | 6/3    |       |   |
| Organism                | Perit. | Blood |   | Perit. | Blood |   |
|                         |        | +++   | + |        | +++   | + |
| E. coli                 |        |       |   | 5      |       | 3 |
| Enterococci             |        |       |   | 1      |       |   |

Figure 4. Bacterial findings in peritoneal fluid and in blood after various periods of hypothermia. Less than 10,000 bacteria per ml blood is denoted as +, more than 10,000 as +++.

# PERITONITIS AND BACTEREMIA IN MICE

- A. Identic findings from blood and peritoneal fluid: B + P  
 Findings from peritoneal fluid when sterile blood: P  
 Findings from blood when sterile peritoneal fluid: B

|  | B + P | P | B        |
|--|-------|---|----------|
| <i>E. coli</i>                             | 27    | 2 | 1        |
| <i>Alcaligenes faecalis</i>                | 1     | 1 |          |
| <i>Proteus</i>                             |       | 1 | 4        |
| <i>Enterococci</i>                         | 2     |   |          |
| <i>B. mesentericus</i>                     | 1     |   |          |
| <i>E. coli</i> + <i>paracoli</i>           |       | 1 |          |
| <i>E. coli</i> + <i>A. aerogenes</i>       | 3     |   |          |
| <i>E. coli</i> + <i>Alcalig. faecalis</i>  | 1     | 2 |          |
| <i>E. coli</i> + <i>Proteus</i>            | 4     | 2 |          |
| <i>Paracoli</i> + <i>Alcalig. faecalis</i> | —     | — | <u>1</u> |
| Total                                      | 39    | 9 | 6        |

- B. Partial identity between peritoneal and blood findings.

|        |                                      | Recovered from blood |                                     |
|--------|--------------------------------------|----------------------|-------------------------------------|
|        |                                      | <i>E. coli</i>       | <i>E. coli</i> +<br><i>paracoli</i> |
| From   | <i>E. coli</i> + <i>paracoli</i>     | 1                    |                                     |
| peri-  | <i>E. coli</i> + <i>A. aerogenes</i> | 1                    |                                     |
| toneal | <i>E. coli</i> + <i>enterococci</i>  | 1                    |                                     |
| fluid  | <i>Paracoli</i> + <i>Proteus</i>     |                      | 4                                   |

Figure 5. Bacterial species recovered from blood and peritoneal fluid in mice sacrificed or succumbed after prolonged hypothermia.

after various periods of hypothermia. Inten animals sacrificed after 24 or 40 hours, the peritoneal fluid as well as the blood was sterile. The findings from the remaining 82 mice are presented in Figure 4, where colony counts below 500 are marked "+" and those above 500 "+++".

From the table it is evident that in mice sacrificed in good condition, peritonitis and bacteremia are fairly infrequent when compared to their occurrence in spontaneously dead animals after long or short periods of hypothermia. In the group which succumbed early bacteria were present in peritoneal fluid in 5 instances without oc-

curring in blood, but in blood only once without being present in the peritoneum.

The degree of correspondence between the bacterial findings from peritoneum and those from blood is visible from Figure 5a. Full conformity existed in 39 cases, whereas in 9 mice, bacteria were present in peritoneal fluid but not in blood, and in 6 instances in blood without being present in peritoneum. Partial conformity existed in the remaining 7 cases.

#### Influence of Hypothermia on the Elimination of Bacteria From the Blood

In a number of preparatory experiments various numbers of E. coli of strains emanating from mouse feces were injected intravenously into normal mice. After different periods, the blood count of live bacteria was measured. The results are reported in Figure 6. Regardless of the number of bacteria injected, the elimination was rapid during the first 15 minutes, bringing down the bacterial counts to about one tenth of the numbers found 4 minutes after the injection. In one experiment, where the first count was made only one minute after the injection, the reduction was equally rapid from this time. After the fifteenth minute, the elimination rate was reduced. This varied somewhat between different experiments, but always resulted in a complete or almost complete elimination. All animals survived.

Considering the frequent occurrence in the blood of E. coli after prolonged hypothermia, some other organism not usually found in endogeneous bacteremia had to be chosen for the elimination studies on hypothermic mice. Hence, the following experiments were performed with Klebsiella pneumoniae in a constant dose of  $4 \times 10^6$  bacteria. The following groups, each containing 5 mice, were studied: normal mice; mice having received premedication but not chilled; mice made hypothermic 5 minutes after the injection of bacteria, 2 hours before the injection, 24 hours (2 groups), and 48 hours (2 groups) before same injection. The results are presented graphically in Figure 7.

## PERITONITIS AND BACTEREMIA IN MICE

log number of bacteria  
per ml blood

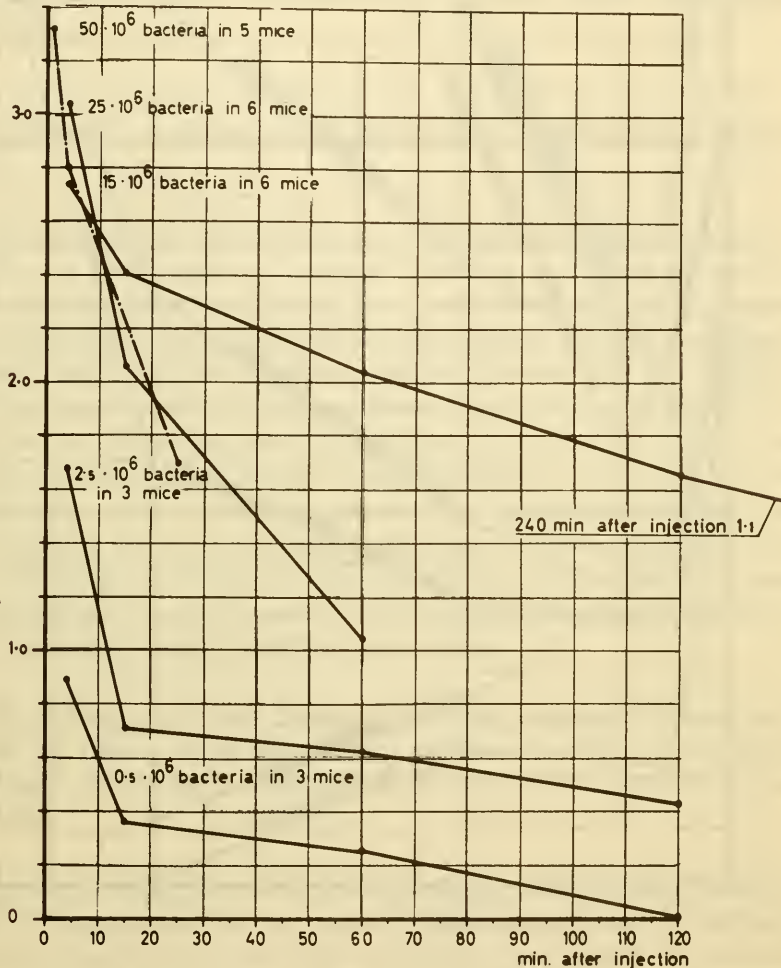


Figure 6. Results of blood cultures in mice at different times after the intravenous injection of *E. coli* suspensions. The points denote logarithmic means. Numbers of mice in each group and number of bacteria injected are given in the figure.

In normal animals the initial elimination was rapid in this series also, leading to complete disappearance of bacteria from the blood. In hypothermic animals the 4 minute counts gave higher numbers which increased with the length of the previous period of hypothermia. Further, the elimination process was interrupted after 2 or



# TUNEVALL AND LINDNER

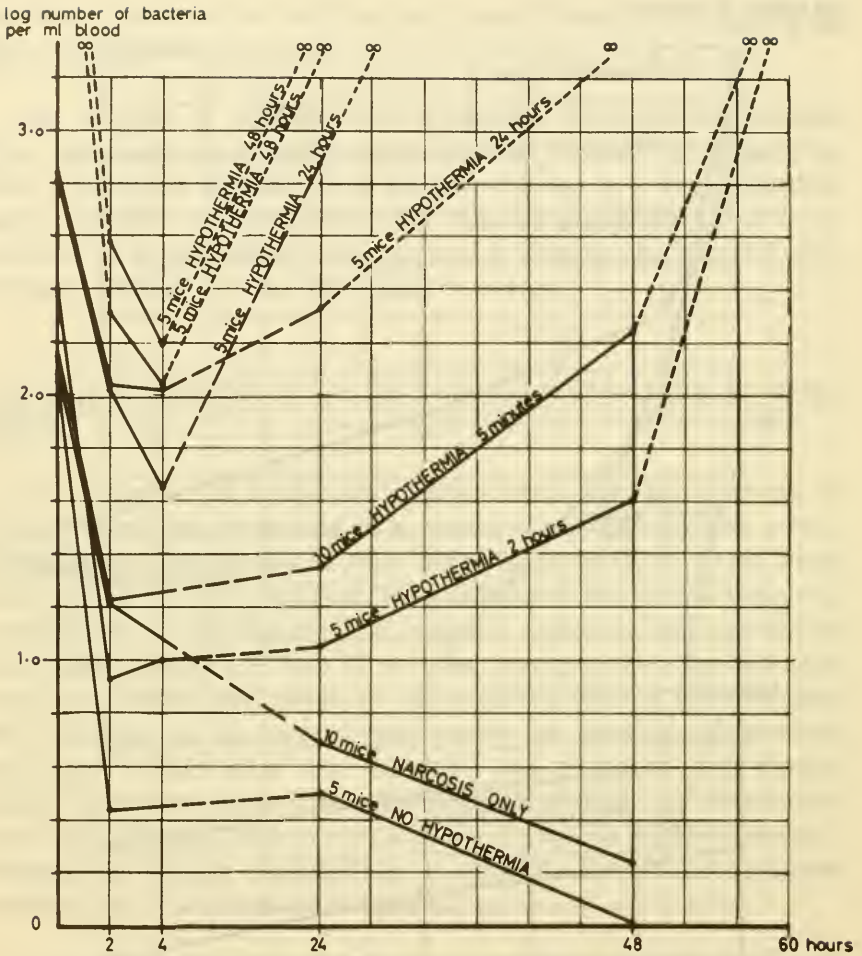


Figure 7. Results of blood cultures in mice at different times after the intravenous injection of  $4 \cdot 10^6$  *Klebsiellae*. The points represent logarithmic means. The numbers of mice in the groups and the length of hypothermia preceding the injection are given in the figure.

4 hours. The level of the minimum colony count also varied with the length of the preceding period of hypothermia. Thereafter an increase of the bacterial counts occurred, ending in a state with innumerable colonies also from the highest blood dilutions. This took place after 72 hours in mice hypothermic 2 hours prior to

injection; after 48 hours in those cooled down 24 hours before; and after 24 hours in mice hypothermic for 48 hours before injection of bacteria.

## The Influence on Endogeneous Bacteremia of Antibacterial Treatment

The experiments now described indicate that bacteremia or peritonitis, or both, occurred almost regularly in mice subjected to prolonged hypothermia, though they were more frequent in mice which succumbed in a hypothermic state than in animals sacrificed in good condition after the same time for hypothermia. The significance of these observations is not clear. Bacteremia and peritonitis may be the dominating cause of debilitation and death, or it may be a concomitant of other changes due to hypothermia. In order to shed some light on this problem, experiments were arranged in which hypothermic mice were treated with a combination of sulpha drugs and streptomycin during hypothermia. This combination was found in preliminary in vitro tests to be most effective against the bacterial strains usually found in bacteremia.<sup>1</sup>

The influence of such treatment on the intestinal flora is presented in Figure 1b, from which it can be seen that a shift from a dominance of coliform bacilli to a prevalence of enterococci was effected.

In several of the previously described experiments on the development of bacteremia in prolonged hypothermia, groups of antibacterially treated mice were run in parallel. The results are seen in Figure 4b and Figures 2b and 3b. It is evident that bacteremia was largely prevented by the treatment. The survival time, however, was not prolonged; not even in the experiment where the untreated animals were given blind injections to compensate for the stress which repeated injections per se might mean for the treated mice.

<sup>1</sup> The preparations and doses used were: "Sulfodital" (Sulfonazole 37 p. c., Sulfadiazine 37 p. c., Sulfamerazine 26 p. c.) 100 mg/kg/day; subcutaneously divided into two doses; and streptomycin 40 mg/kg/day, in one dose.

## DISCUSSION

Observations of other investigators, corroborated in the present study by findings to be discussed below, make us accept the view of the intestinal flora as the source of endogeneous bacteremia in prolonged hypothermia. The ability of intestinal bacteria to invade the rest of the body during the hypothermic state might be due to a change of this flora itself. The absence of food intake, the slowing down of intestinal motility, and the low temperature seem likely to effect such a change. However, as shown in Figure 4, no major alteration of the composition occurred. It must be stated in this connection that our studies were restricted to aerobic organisms.

By repeated blood cultures in hypothermic mice, bacteremia was found to be an almost constant result of deep and prolonged hypothermia, as shown in the Figures 2 and 3. It should be noted that no bacterial type was ever demonstrated in the blood without having been found previously in the intestine. Intestinal and blood findings were not compared by serological typing but in biochemical reaction of *E. coli* strains and in Dienes' identity test for *Proteus bacilli* (Dienes, 1946) full conformity was found between every pair of strains tested.

As to the route of the bacterial invasion from the intestine, Figures 4 and 5 indicate that in sacrificed or succumbed mice, bacteria were more often found in peritoneal fluid without being present in the blood than in blood without being found in the peritoneal cavity. Thus, the invasion often started with a peritonitis but seemed also to take place directly into the lymph or blood stream. Hammond et al., (1954) refer to experiments where bacteria were found to penetrate the intestinal wall and to reach the mesenteric lymph nodes in X-irradiated mice.

A demonstrable occurrence of bacteria in the blood can, apart from an abnormal import as suggested by Billingham (1957), be due to a diminished ability of the organism to eliminate bacteria from the blood stream. Our experiments reported in Figure 7 demonstrate such an impaired elimination; the more it was decreased, the longer



## PERITONITIS AND BACTEREMIA IN MICE

the hypothermic state existed. They also show that the intravenous injection of bacteria which are normally eliminated from the blood without any consequences for the animal results in a profuse bacteremia in mice in deep hypothermia. We have not yet had the opportunity to investigate whether this disagreement with Fedor's (1958) results are due to differences as to the kind of animal, the degree of hypothermia, or its duration, but it may be mentioned that the observations of Gowen and Friou (1961) in dogs tally with our results in mice.

The observation that peritonitis and bacteremia are more frequent in spontaneously dead mice than in animals sacrificed in good condition after the same time of hypothermia may be interpreted in two ways. The generalized infection may be a dominating cause of death, or it may be a concomitant phenomenon to other injuries caused by hypothermia. In the former case it should be possible to diminish the mortality of hypothermic mice with antibacterial treatment. Such treatment was given to groups of hypothermic mice.

The treatment (streptomycin + sulpha) effected a pronounced change in the aerobic intestinal flora from a preponderance of the *Escherichia-Aerobacter* group to a dominance of enterococci. The latter species, however, did not replace the Gram-negative rods as blood invaders. Generally, no bacteremia was observed in treated animals. However, the average survival time in prolonged hypothermia was not increased. Thus, bacteremia seems not to be a determining factor in the debilitation and death of hypothermic mice. These observations coincide, for example, with those recorded in irradiated mice by Laurell et al. (1958) and Fallowfield et al. (1962), but differ from the results of Miller et al. (1952).

### II. Experimental Peritonitis and Bacteremia

Many studies have dealt with the effect of low temperature on induced infection. The results have probably been contradictory be-



cause of differences in the choice of experimental animals and infecting micro-organisms, and also of the mode of exposure to cold. Thus, in one type of study animals have been exposed to cold under conditions that provoke stress reactions, and chilling has generally been found to increase the susceptibility. In 1878, Pasteur found that chilled fowl became susceptible to anthrax. Increased susceptibility to trypanosomes at low environmental temperature has been seen in guinea pigs (Kligler and Weitzmann, 1926); in rats (Kligler, 1927) and mice (Kligler and Olitzki, 1931) to Bac. enteritidis; and in rabbits to streptococci (Carpano, 1926) and to syphilis (Longhin et al., 1957). Other such experiments have shown lowered resistance of chilled guinea pigs to pseudotuberculosis but not to pneumococci (Burgers, 1929), indicating the importance of the choice of infecting organism is important. This was also borne out by studies of Previte and Berry (1962) in which chilled mice were abnormally sensitive to relatively avirulent *Salmonellae* and staphylococci but normally susceptible to virulent strains. It is noteworthy that Bruneau and Heinbecker (1944) found that in dogs inoculated subcutaneously with  $\beta$ -streptococci, cooling reduced the local inflammatory reaction and arrested bacterial growth. On removal from the cold, however, both these processes were abnormally accelerated, indicating that prolonged chilling had in the long run lowered the resistance.

In another type of experiment, body temperature has been lowered in a way which avoids stress reactions. The results have been different in that infection with virulent Type I pneumococci in rabbits was not changed by hypothermia, whereas infection with normally avirulent Type III pneumococci became lethal in hypothermic animals (Muchenheim et al., 1943). Similar observations as to the result of infection with pneumococci of low virulence in rabbits were made by Sanders et al. (1957), but they also found a decrease of the survival time in virulent infection. On the contrary, Wotkyns et al., (1958) working with mice found a longer survival time in hypothermic animals with Type III pneumococcal peritonitis suggesting defense mechanisms. Similar observations on rats with peritonitis were made by Balch et al. (1955), and on rabbits with staphylococcal sepsis by Grechishkin (1956).

# PERITONITIS AND BACTEREMIA IN MICE

## EXPERIMENTAL

### Method

Pneumococcal infection was induced by intraperitoneal (i.p.) injection of broth cultures in doses which were previously found to cause infection of a desired course and massivity in normal mice.

### Results

In preliminary experiments for stating optimal conditions for the following study, hypothermia was found generally to prolong the average survival time of mice after their inoculation (i.p.) with pneumococci. The results of such an experiment are reported in Figure 8.

In all, 32 mice were inoculated (i.p.) with 20,000 pneumococci Type III, while six mice were kept as controls. About three hours later, 16 inoculated mice and the controls were made hypothermic. The remaining 16 inoculated mice were kept normothermic. After about 49 hours, surviving hypothermic mice were warmed up. Cultures from heart blood were made from succumbed mice as soon as we were sure they were dead.

The average survival time in the hypothermic group was 36 hours as opposed to 16 hours in the normothermic group and 59 hours for five out of six hypothermic controls not inoculated; the sixth animal in this group survived. In the inoculated hypothermic group, three mice lived to be warmed up, but died during or soon after this procedure. Pneumococci were found in peritoneal fluid of all inoculated mice. In heart blood they were abundant in all normothermic mice, but only in six hypothermic ones, whereas three had pneumococci in moderate numbers and seven mice had even less.

It should be added that among five inoculated hypothermic mice and five not inoculated ones which sustained hypothermia

# TUNEVALL AND LINDNER

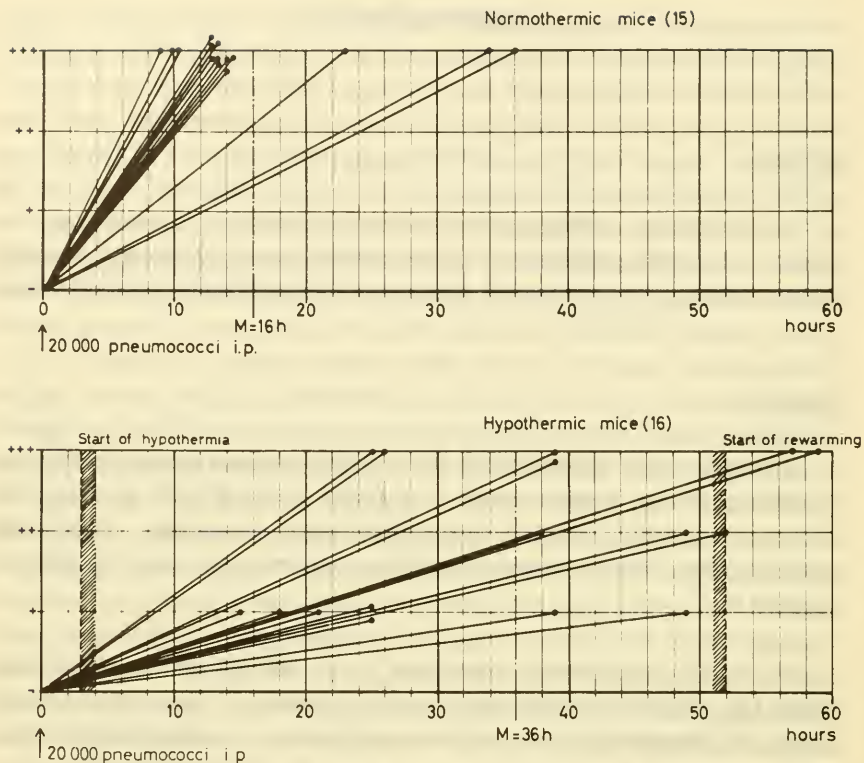


Figure 8. Results of post mortem blood cultures in normothermic mice and hypothermic ones given 20,000 pneumococci Type III intraperitoneally. + = 1-50 colonies, ++ = 51-500, +++ = more than 500.

for 46 hours or more, seven animals had coliform bacteria in blood and/or peritoneum.

The first experiment was only semi-quantitative and indicated just the final result of the infection, because post mortem cultures only were made. In the following study the events were followed more closely.

In the experiment reported in Figure 9, 15 mice were made hypothermic while another 15 received the pretreatment only. All mice were inoculated (i.p.) with 200,000 Type II pneumococci eight

# PERITONITIS AND BACTEREMIA IN MICE

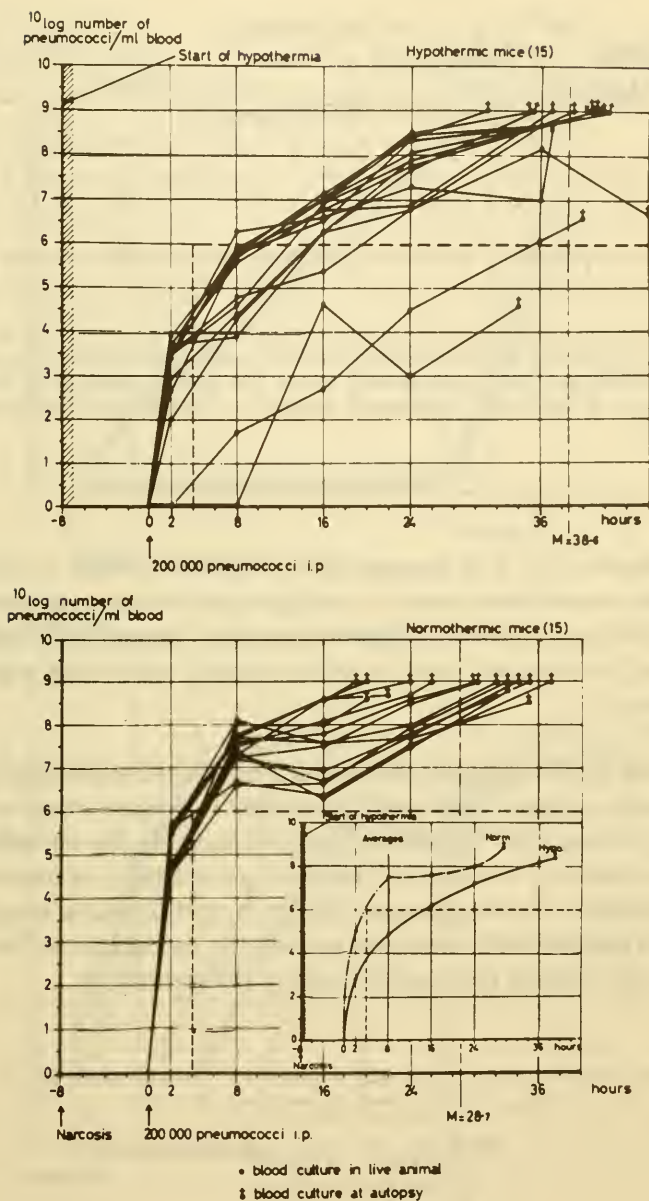


Figure 9. Results of intra vitam and post mortem blood cultures in hypothermic mice and normothermic ones given 200,000 pneumococci Type II intraperitoneally. Average curves inserted below.



# TUNEVALL AND LINDNER

| Hours after<br>inoculation | M <sup>10</sup> log nr of pneumococci<br>± mean error of means |              | Diff. | t    | P      |
|----------------------------|--|--------------|-------|------|--------|
|                            | Hypothermic  | Normothermic |       |      |        |
| 2                          | 2.91 ± 0.33  | 5.00 ± 0.12  | 2.09  | 6.15 | <0.001 |
| 8                          | 4.71 ± 0.45  | 7.50 ± 0.11  | 2.79  | 6.18 | <0.001 |
| 16                         | 6.25 ± 0.31  | 7.63 ± 0.22  | 1.38  | 3.73 | <0.001 |

Figure 10. Number of pneumococci in blood at different times after i. p. inoculation in hypothermic and normothermic mice. The figures are given as <sup>10</sup>logs, with mean errors of the means. Differences, t-values in Student's test, and P-values are also given.

hours afterwards. The hypothermic mice were kept in this state during the whole experiment. In both groups blood counts were made 2, 8, 16, 24, and 36 hours after inoculation. Succumbed mice were autopsied immediately and bacterial counts were then made from heart blood.

As seen from the figure, bacteremia increased more slowly in the hypothermic mice. This also presented a longer survival time;  $38.8 \pm 1.0$  hours as against  $28.7 \pm 1.6$  hours for the normothermic ones (t-value 5.5,  $P < 0.001$ ). The average numbers of pneumococci per ml blood in the two groups after 2, 8, and 16 hours are given in Figure 10 together with results of probability calculations. The formula used for testing the significance of differences is:

$$t = \frac{M_x - M_y}{\sqrt{\frac{(\bar{x}-x)^2}{n_x} + \frac{(\bar{y}-y)^2}{n_y}}}$$

From the figures in the table, the slower increase on the bacterial numbers among hypothermic mice is verified. After the 16th hour, the differences are no more significant.

# PERITONITIS AND BACTEREMIA IN MICE

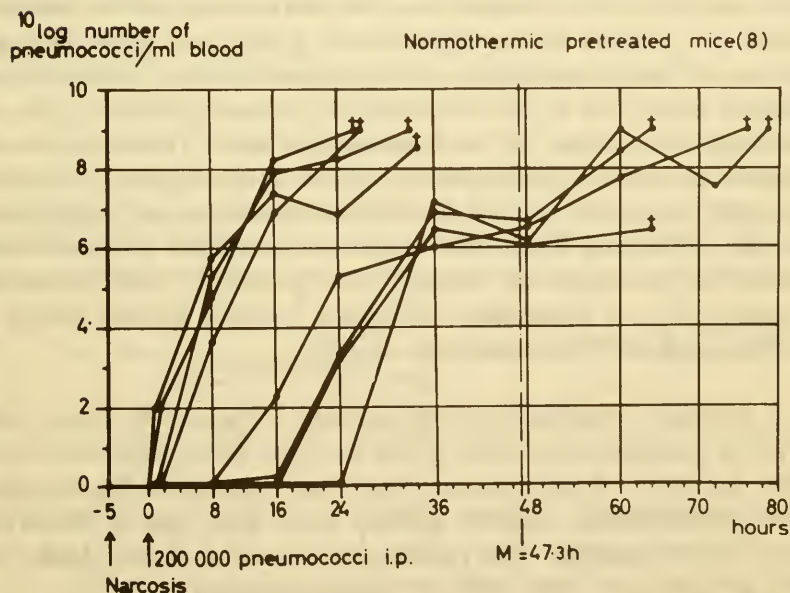
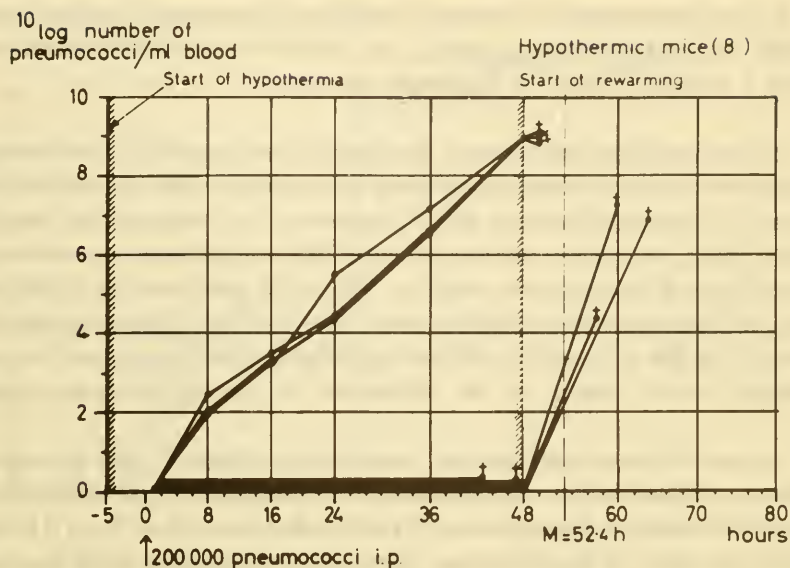


Figure 11. Results of inter vitam and post mortem blood cultures in hypothermic mice and normothermic ones given 200,000 pneumococci Type II intraperitoneally. Markings as in Figure 2.

## TUNEVALL AND LINDNER

It was interesting to note that hardly any pneumonic lesions were found in the hypothermic group, but in five normothermic mice all with a survival time of 30 hours or more.

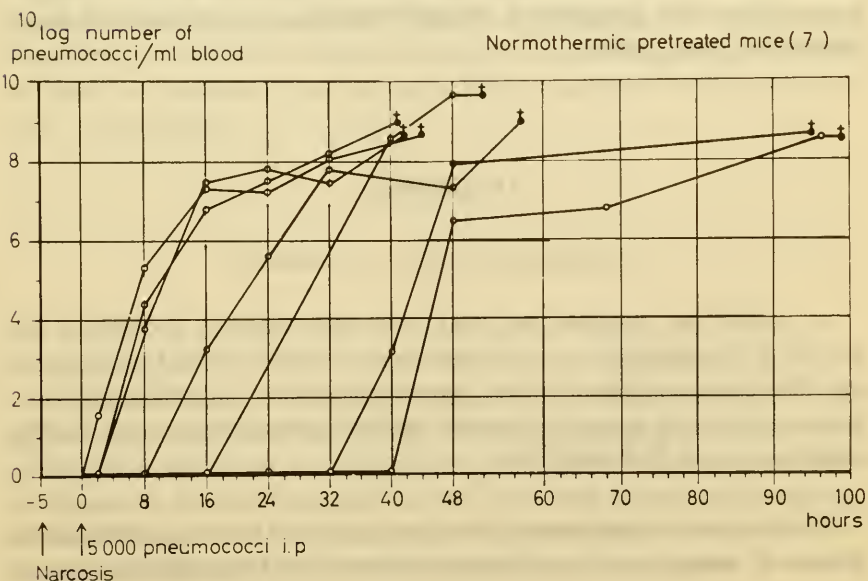
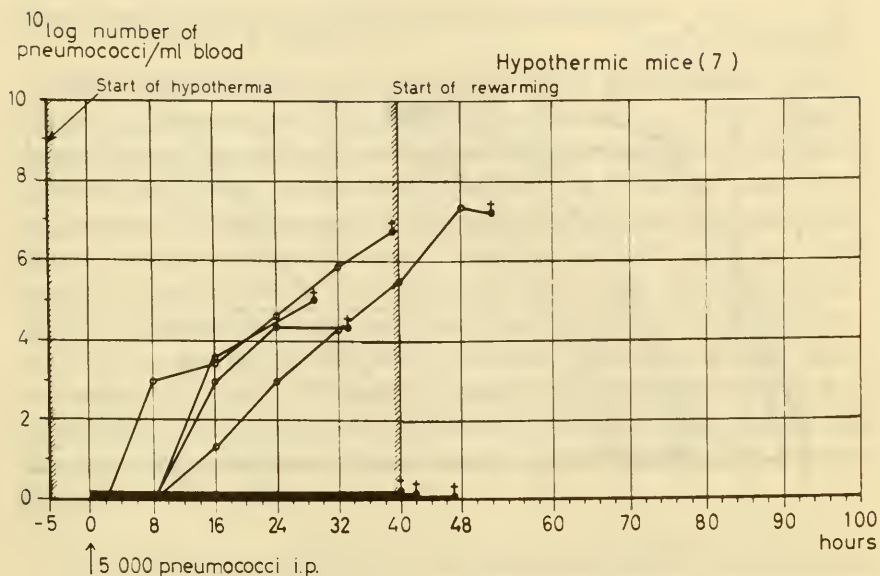
In the previous experiment the inoculation resulted in bacteremia which was always established after two hours in the normothermic group. In the hypothermic group, however, two animals had sterile blood after two hours and one of them after eight hours. In order to investigate if bacteremia could be regularly postponed by hypothermia, a less massive infection was induced in the following experiments, as the propagation of such an infection into the blood stream seemed more likely to be influenced by the hypothermic state.

In one of these experiments, presented in Figure 11, the hypothermic as well as the normothermic group contained eight mice. Two hundred thousand pneumococci Type II were inoculated (i.p.) 5 hours after the start of hypothermia. Hypothermic animals were warmed up 48 hours after the inoculation. Also, the increase of the bacterial counts was slower in the hypothermic group, and after as long a time as 40 hours, bacteremia had developed in only 3 of the 8 hypothermic mice, but in all normothermic animals. However, the re-warming was noxious to the hypothermic mice. Three bacteremic animals already in the hypothermic state died during this procedure; two other mice died as well, but without developing any bacteremia; and the remaining three died within 15 hours from the start of the rewarming procedure and proved to be bacteremic when autopsied. Consequently, no significant difference between the two groups as to the mean survival time was found.

A similar experiment is presented in Figure 12. Here, 5,000 Type II pneumococci were given (i.p.) to seven mice five hours after the start of hypothermia which was maintained for 40 hours. Two normothermic control groups were run, one of which was given premedication. The results were similar within both control groups, and they will be treated together.

The results tallied well with those of the previous experiment. Bacterial multiplication was slower in hypothermic mice, and three such mice were protected from bacteremia, whereas all controls became bacteremic. Rewarming was fatal. All surviving mice

# PERITONITIS AND BACTEREMIA IN MICE





## TUNEVALL AND LINDNER

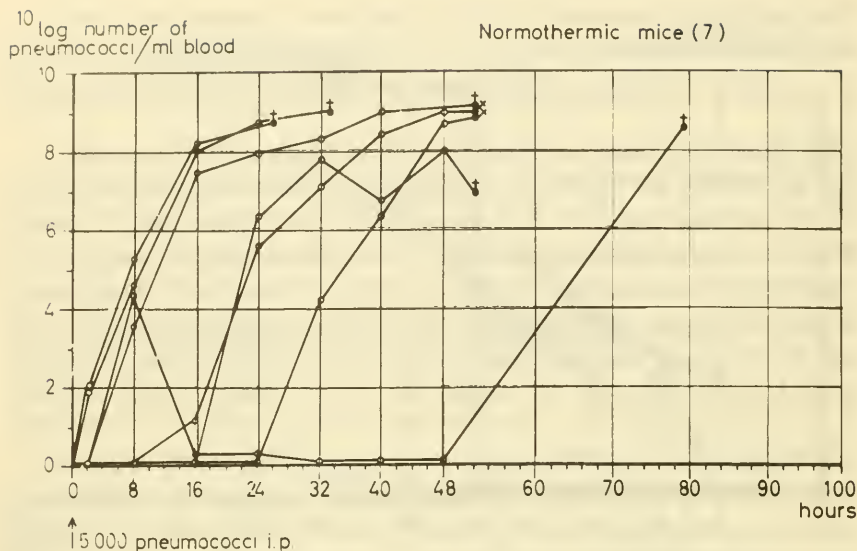


Figure 12. Results of inter vitam and post mortem blood cultures in hypothermic mice and normothermic ones given 200,000 pneumococci Type II intraperitoneally. Markings as in Figure 2.

died; one at start of rewarming, and the remaining two within 12 hours after this procedure. No difference as to the survival time, therefore, was noted.

## DISCUSSION

It should be pointed out that our experimental procedure for attaining hypothermia was devised to decrease the body temperature by elimination of the normal thermoregulation at chilling. Thus, stress reactions were minimized and body temperature was rapidly stabilized at 22° C to 23° C.

Preliminary experiments indicated a slower increase of the blood counts of pneumococci and a prolonged survival in hypothermic mice

## PERITONITIS AND BACTEREMIA IN MICE

after the induction of pneumococcal peritonitis.

These observations were corroborated by experiments in which bacterial counts from blood were done repeatedly. In other experiments where the infection was less massive the slower course of disease was found to manifest itself in two different ways. First the invasion of pneumococci from the peritoneal cavity to the blood stream was more often delayed in hypothermic animals. If the fairly similar experiments of the Figures 11 and 12 are taken together, 7 out of 15 hypothermic mice were bacteremic 40 hours after the inoculation as against 20 out of 22 controls (Chi-square with Yates' correction 6.9;  $0.01 > P > 0.001$ ). Second, when bacteremia was once established, the increase of the number of bacteria in the blood was slower in hypothermic mice. Our results thus tally with those of Watkyns et al. (1958).

But one other significant observation was made; warming up of hypothermic mice was deleterious. Bacteremic mice died during or soon after this procedure and in mice which still had sterile blood, bacteremia was often established and followed by death within a short time. In others, death occurred without being preceded by bacteremia. It is not possible to decide whether this effect was caused by a weakening of the resistance by hypothermia against bacterial assault as a whole or, more specifically, to an increased sensitivity to bacterial toxins during the rewarming process which in itself is traumatic and may involve an abnormal acceleration of cell metabolism.

## SUMMARY AND CONCLUSION

I. Experiments with hypothermic mice maintained at 22° C to 23° C for more than 40 hours, almost invariably resulted in the emergence of bacteria in peritoneal fluid and blood. These bacteria belonged to species present in the intestine. When peritoneal and blood findings were not identical, the demonstration of a species in peritoneal fluid only was more frequent than in blood only. No major

change of the intestinal flora was effected by the hypothermia.

The elimination from the blood stream of injected bacteria was found to be disturbed in hypothermic animals, and even to a greater extent in long periods of hypothermia. *Klebsiellae* rapidly eliminated in normal mice diminished in number only during a period of 2 to 4 hours, but thereafter increased again and caused profuse bacteremia in hypothermic mice.

Treatment with a sulpha-streptomycin combination provoked a shift of the intestinal flora towards a dominance of enterococci and diminished considerably the incidence of bacteria in peritoneal fluid and in blood, but did not influence the survival time of hypothermic mice.

It can be concluded that deep and prolonged hypothermia in mice results in an invasion of bacteria from the intestine into the peritoneal cavity or into the blood stream or both. This invasion is not due to any change of the intestinal flora, but may be contributed to by an inability of hypothermic mice to eliminate bacteria from the blood stream. As antibacterial treatment reduced the incidence of peritonitis and bacteremia but did not increase the survival time of hypothermic mice, the bacterial invasion is not likely to be any important factor for debilitation and death, but rather a concomitant phenomenon.

II. Experimental pneumococcal peritonitis was found not to result in bacteremia as regularly or as early in hypothermic mice as in normothermic controls. Once established, the bacteremia also increased in massivity more slowly in hypothermic mice which also had longer average survival time.

Warming up of the hypothermic mice resulted in a rapidly increasing bacteremia and death, or in death not preceded by bacteremia. This might be due to a diminished resistance as a result of the previous hypothermia, to the trauma inherent in the rewarming procedure, or to an increased susceptibility to bacterial toxins during this process.

# PERITONITIS AND BACTEREMIA IN MICE

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## DISCUSSION

MIYA: How soon after the mouse succumbed did you take your blood cultures?

TUNEVALL: When it occurred in daytime, it was made as soon as we were sure that the mouse was dead. If it occurred during the night, if someone were in the lab, the procedure was the same one. If we were not -- we had to sleep now and then -- a technician would take the mouse directly to the deepfreeze

## PERITONITIS AND BACTEREMIA IN MICE

and chill it down there. Then it was cultured the following day.

MIYA: Is it possible that some of your positive blood cultures were just due to the breakdown of your normal tissue barriers before you got the blood?

TUNEVALL: Maybe this could not be entirely excluded, but we have done some model experiments to see if this treatment of the dead mouse very often results in growing through from the intestine, but we have not the impression that this factor has disturbed the results very much.

MARCUS: Yesterday, when the speaker spoke about antibody formation and measurement in rabbits, he spoke about it in rabbits using quantitative antibody estimations by classical methods of determination. He did not consider the possibility of formation of non-precipitating antibody, and I think that this is probably valid because most rabbits don't ordinarily form too much of this. But you are using mice, and I wondered if this might not be a significant consideration. I don't know what the system was that you employed for measuring the antibody, but I wondered if you had given it any consideration at all?

TUNEVALL: We used the typhoid-H antigen, and for determining the antibody level, we simply used the Widal test.

MARCUS: One other thing I wanted to ask about. In discussing the mechanism of invasion of the hypothermic mouse, in comparing it to what occurs in the irradiated animal, there are some interesting possibilities. As far as I know, no one knows the molecular lesions that occur following irradiation which lead to invasion, but there is good evidence at the cellular level of what the lesions and resistance are. The mouse does not have any bactericidal component in its serum, but following irradiation there is not a loss of phagocytic capacity, but a loss of intracellular destructive capacity, and I wonder if this is the same type of lesion which might occur in the hypothermic mouse.

TUNEVALL: I'm sorry, I cannot answer that question.



## TUNEVALL AND LINDNER

MARROW: I will describe two clinical observations. An infant with considerable cold exposure, due to drunkenness of the parents, was brought into the hospital, failed to respond to massive antibiotic therapy, and the youngster died with peritonitis, which would be exactly parallel to your experiments taken over into a human, although the degree of lowering of body temperature, we do not know. The parents were inebriated to the point of anesthesia for a period of a couple of days, so we don't know how long the youngster was hypothermic. We have had six hypothermic, or, let's say, "sub-normal temperature" individuals below the age of three months. The first two were treated conventionally with massive doses of penicilin, both dying within the third or fourth day of hospitalization. The next four were given massive doses of gamma globulin immediately upon admission, and had relatively uneventful recovery.

# THE ROLE OF LOW ENVIRONMENTAL TEMPERATURES IN PREDISPOSING MICE TO SECONDARY BACTERIAL INFECTION<sup>1</sup>

Gennaro J. Miraglia<sup>2</sup> and L. Joe Berry

## ABSTRACT

The LD<sub>50</sub> for mice of S. typhimurium, strain RIA, is  $4.1 \times 10^5$  cells for animals individually housed without bedding and maintained at 25° C. It is  $3.8 \times 10^3$  cells for animals similarly housed but kept at 5° C. However, mice are able to withstand nearly 100 times this dosage of strain RIA if they are housed in groups at 5° C. Normal mice with their dorsal and ventral surfaces shaved are unable to survive more than a few days when housed individually in the cold, but survive beyond two weeks under group housing conditions. No effect of cold could be detected in mice infected with the highly virulent SR-11 strain of S. typhimurium since all animals died following infection with only a few cells. Mice that were natural carriers of salmonellae as judged by fecal discharge were highly resistant to challenge and responded to cold in a manner similar to normal mice infected with RIA. Strain RIA could be isolated from the tissues of infected animals with greater frequency and persisted longer in mice maintained at 5° C than those at 25° C. Coagulase negative staphylococci were isolated from liver, lung, spleen, heart, and kidney of animals that survived salmonellosis for 14 days at 5° C. The staphylococci did not appear to have a predilection for one tissue over another, and were isolated in an incidence proportional to the number of salmonellae injected in the primary infection. At 25° C, only a small percentage of mice had staphylococci in tissues, and these occurred independent of the infectious dose of salmonellae. These observations were made on normal mice infected with RIA and on carrier mice infected with SR-11. The origin of the secondary invader remains obscure, but it does not appear to result from a penetration of coagulase negative staphylococci from the lumen of the gut to the deep tissue. Mice devoid of intestinal staphylococci and recolonized with coagulase positive staphylococci continue to show coagulase negative isolates from deep tissue.

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The extent to which exposure of experimental animals to low environmental temperature alters or modifies the outcome of host-parasite interaction is not clearly established. Pasteur, nearly a century ago, attributed the resistance of chickens to anthrax to the characteristically high body temperature of fowls. By inducing hypothermia he was able to render them lethally susceptible. In the intervening years, reports dealing with the effects of cold on the course of bacterial infections have been few in number. On the other hand, literature concerned with the physiological effects of exposure to low temperatures on various mammalian species is extensive, including several reviews (Hemingway, 1945; Hardy, 1950; Hardy, 1961; and Smith and Hoijer, 1962).

There are, nevertheless, important studies on the contribution of the environment to the response of animals challenged with several different infectious agents. Junge and Rosenthal (1948), for example, studied the survival of mice infected with pneumococci and reported increased susceptibility when the temperature was decreased to 18° C. It was necessary, however, to treat the animals with sulfadiazine immediately following infection in order to insure sufficient survival to make the temperature effect apparent.

Muschenheim and collaborators (1943) had studied several years earlier the effect of hypothermia in rabbits on resistance to experimental pneumococcal infection. When a highly virulent strain was employed, the only demonstrable effect of lowered body temperature on host response to the pathogen, compared to that in normothermic animals, was a decrease in the local inflammatory reaction. When a strain of low virulence was used, the induced hypothermia resulted in bacteremia and death in addition to the inhibition of the dermal inflammatory reaction.

The interaction between certain viruses and a variety of hosts as influenced by environmental temperature has received considerable attention in recent years. The incisive investigations of Boring et al. (1956) are particularly germane to this paper. Cold was found to have an adverse effect on the mouse infected with a Cosxackie virus. The animals were housed 8 to 12 per cage at 4° C without restriction on huddling. At this temperature there was a viremia through the fourth post-infection day while the blood was clear of virus by that

## SECONDARY BACTERIAL INFECTION IN MICE

time in mice at 25° C. Similarly, the titer of virus in the liver was higher on the fourth day in mice at 4° C than in mice at 25° C. These experiments indicate that although adult mice possess a natural resistance to the Coxsackie virus such that the disease is limited to a non-fatal infection, this resistance is lost when animals are maintained in the cold. Under these conditions, a lethal infection ensues which is characterized by a persisting viremia, high levels of virus in the liver, and lesions demonstrable in other organs. The mechanism by which cold reduces resistance to the virus is unknown, although the fact that cortisone causes a similar loss of resistance suggests that cold may act through its capacity to augment secretion of adrenocortical steroids. It may also result in the involvement of other body responses less well defined. Thus, Walker and Boring (1958) observed that neutralizing antibody appeared on the fourth day in mice at room temperature but failed to appear in animals in the cold. Injections of cortisone are known to suppress the immune response (Germuth, 1956).

Schmidt and Rasmussen (1960) reported that mice maintained at 37° C were more resistant to infection with herpes simplex virus than those held at 25° C. This protective effect was believed to be due to the lower viral population in brain tissue at the higher temperature. The mechanism responsible for this decrease in the number of viruses is unknown, but a possible explanation for the difference in mortality rates is an alteration in viral multiplication due to a temperature induced change in the metabolism of host tissues. It has been well established that viral populations can be controlled to some extent by only a few degrees change in temperature (Lwoff, 1959).

The object of the present study was to determine possible differences in the course of salmonellosis in mice maintained at 25° C with others kept at 5° C, and to uncover, if possible, mechanisms responsible for such differences. It was not our intention, however, to employ mice with a controlled hypothermia. In the first place, the lower environmental temperature to which animals were subjected failed to depress the core temperature below the normal range. In addition, to attempt to regulate the degree of hypothermia in populations of mice the size of those used in the experiments would have been technically beyond the resources available. The ability of mice



## MIRAGLIA AND BERRY

to maintain normothermia when kept at 5° C for 10 to 15 days has been reported previously by Bischoff (1942). Nevertheless, moribund animals in the cold do become markedly hypothermic, as they do during the last hours of life at room temperature when suffering from salmonellosis.

The data to be presented confirm and extend those of Previte and Berry (1962) who demonstrated an increase in host susceptibility to infection with Salmonella typhimurium when a strain of low virulence but not one of high virulence was used in mice continuously exposed to a low ambient temperature. In addition, and perhaps of even greater importance, is the observation that a second invading organism, a coagulase-negative staphylococcus, presumably from the environment or from the mouse itself, becomes established in tissues of the host already stressed by cold and the experimental salmonellosis. It has long been believed that primary infections predispose an animal to secondary invasion but, heretofore, experimental evidence for this has not been clear. To the authors' knowledge, this is the first description of a situation which permits consistent prediction of the incidence of secondary infection.

## MATERIALS AND METHODS

Animals. CF<sub>1</sub> female mice (Carworth Farms, New York City, New York) weighing 20 to 22 gm were used in all experiments. They were housed in plexiglass cages which were divided into 10 equal compartments per cage. Each compartment measured 3.5 x 7.5 x 9.0 cm. No bedding was used, and mice were housed individually to prevent huddling. The open tops and bottoms of the plexiglass enclosures were covered with 3/8 inch mesh hardware cloth. All completely assembled cages were placed on wire mesh to keep the animals free from excessive moisture and excreta. Water and pathogen-free mouse food (Dietrich and Gambrill, Frederick, Maryland) were available at all times.

Animal Rooms. Two animal rooms were used; one was maintained

## SECONDARY BACTERIAL INFECTION IN MICE

at  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ , the other at  $5^{\circ}\text{C} \pm 1^{\circ}\text{C}$ . An automatic lighting system provided 12 hours of light for each 24 hour period. The mice were kept continuously in the appropriate room for the entire experimental period. Humidity was not controlled but results were reproducible at different seasons of the year when humidity is known to vary.

Inoculum. Two strains of Salmonella typhimurium were used, the highly virulent SR-11 and the relatively avirulent RIA. Both strains were cultivated in brain-heart infusion broth (Difco) for 16 hours at  $37^{\circ}\text{C}$ . Decimal dilutions of the cultures were prepared with pyrogen-free saline (Baxter Laboratories, Morton Grove, Illinois) and were plated in triplicate on both nutrient and SS agar plates (Difco) to enumerate the colonies and to insure the uniformity of the culture. Inoculations consisting of the appropriate number of organisms contained in 0.5 ml of saline were administered by the intraperitoneal route.

Organ culture technique. Tissues (heart, lung, kidney, spleen and liver) selected for bacterial culture were excised by aseptic technique from mice immediately after death. Samples were cultured on appropriate media for identification of microflora, using the organ print method. Nasal cultures were also desired, but owing to the obvious difficulties in attempting to obtain inocula from the nasopharynx, only the external nares of the animals were cultured by the imprint method on appropriate medium for staphylococci.

Stool culture technique. Stool cultures were made, where indicated, by placing into the appropriate medium a single fecal pellet obtained on a sterile swab at the time of defecation. To culture for staphylococci, a pellet was incubated at  $37^{\circ}\text{C}$  in brain-heart infusion broth containing 7 per cent sodium chloride for 16 to 18 hours before streaking on Staphylococcus 110 medium (Difco). To culture for salmonellae, a second specimen was similarly incubated in selenite broth (Difco) before streaking on SS agar.

Coagulase test. The coagulase test was conducted in Wassermann tubes using 0.5 ml of reconstituted coagulase plasma (Difco) to which was added two drops of a 16 hour brain-heart infusion culture of the staphylococcus under test. Tubes were read after three hours incu-

bation at 37° C.

Miscellaneous. In lieu of drinking water, 0.01 N hydrochloric acid (pH 2.0) was given to mice to rid the gut of staphylococci. This is a modification of the method of Schaedler and Dubos (1962). The absence of the organisms was confirmed by stool culture. Where recolonization of the intestine with a specific strain (in this case Staphylococcus aureus, strain Giorgio) was desired, the hydrochloric acid treatment was terminated before feeding the mouse for a period of 12 hours the desired microorganism as a contaminant in the ration.

## RESULTS

Determination of LD<sub>50</sub>. The LD<sub>50</sub> for animals infected with strain RIA and maintained at 5° C and 25° C was determined by the method of Reed and Muench (1938). This was found to be  $4.1 \times 10^5$  cells per mouse at 25° C and  $3.8 \times 10^3$  cells per mouse at 5° C, as shown in Table I. The LD<sub>50</sub> for the highly virulent SR-11 strain was less than seven cells per mouse at room temperature, hence a temperature effect was not demonstrable. All observations were terminated after a period of 14 days.

The space limitation imposed on mice housed in the compartmented cages did not alter the LD<sub>50</sub> dose of the RIA strain of S. typhimurium at 25° C. This was true also for the LD<sub>50</sub> dose of crude bacterial endotoxin administered by intraperitoneal injection. Neither crowding nor the psychological effects of isolation under a situation where neighboring mice were visible through the clear plexiglass made any measurable difference in these animals compared to those normally housed.

The same type of control experiment could not be conducted, however, at 5° C since animals permitted to huddle are not as severely stressed by cold as those kept in isolation. This can be seen from the following experiment. Animals were housed 10 per cage (10 x 7 x 6 1/2 inches) with pine shavings as bedding and without restriction

## SECONDARY BACTERIAL INFECTION IN MICE

Number of mice dead/total tested in mice group housed at:

| 5° C |     | 25° C |       |
|------|-----|-------|-------|
| 6/10 | 60% | 17/30 | 56.6% |

Table I. The LD<sub>50</sub> dose of S. typhimurium for mice as influenced by bacterial strain and environmental temperatures.

| Inoculum     | Temperature | LD <sub>50</sub>  |
|--------------|-------------|-------------------|
| Strain RIA   | 25° C       | $4.1 \times 10^5$ |
| Strain RIA   | 5° C        | $3.8 \times 10^3$ |
| Strain SR-11 | 25° C       | 7 cells           |
| Strain SR-11 | 5° C        | 7 cells           |

Table II. Effect of group housing on survival of mice at 5° C and 25° C infected with  $4.8 \times 10^5$  cells of S. typhimurium, strain RIA. Single housing, LD<sub>50</sub> =  $3.8 \times 10^3$  RIA at 5° C. Single housing, LD<sub>50</sub> =  $4.1 \times 10^5$  RIA at 25° C.

on huddling or activity. They were inoculated with  $4.8 \times 10^5$  cells of strain RIA and for those maintained at 25° C, 17 out of 30 (56.6 per cent) died (Table II). This was the anticipated result since this inoculum is the approximate LD<sub>50</sub> for mice at room temperature. On the other hand, mice similarly housed and inoculated and placed at 5° C showed six out of 10 deaths (60 per cent) in response to an inoculum that is characteristically 100 times the LD<sub>50</sub> for animals housed individually in the cold. From these findings it is apparent that cold modifies the response of mice to infectious challenge only under specific conditions of exposure. This is important to keep in mind in comparing the experiments described here with those reported elsewhere.

In another experiment utilizing 20 normal mice, 10 were shaved on their dorsal and ventral surfaces and housed individually; the remaining 10 were also shaved but were group housed with pine shavings as bedding. In 24 hours, eight of the 10 mice housed individually died and all were dead by the fifth day. The group housed mice, however, lived for the entire 14 day experimental period and were then sacrificed. This points up once again the importance of housing conditions in experiments on cold.



## MIRAGLIA AND BERRY

Number of S. typhimurium injected intraperitoneally into mice kept the temperatures indicated

| Days Post Infection | $4.8 \times 10^2$ |                | $4.8 \times 10^3$ |                 | $4.8 \times 10^4$ |                 | $4.8 \times 10^5$ |                 |
|---------------------|-------------------|----------------|-------------------|-----------------|-------------------|-----------------|-------------------|-----------------|
|                     | 25°               | 5°             | 25°               | 5°              | 25°               | 5°              | 25°               | 5°              |
| 1                   |                   |                |                   |                 |                   |                 |                   |                 |
| 2                   |                   |                |                   |                 | 1                 |                 |                   |                 |
| 3                   |                   |                |                   |                 | 1                 |                 | 2                 |                 |
| 4                   |                   |                |                   |                 |                   |                 | 4                 |                 |
| 5                   |                   | 1              |                   |                 | 1                 |                 | 3                 | 5               |
| 6                   |                   |                |                   | 2               |                   |                 | 5                 | 2               |
| 7                   |                   | 3              |                   | 3               | 1                 |                 | 1                 | 3               |
| 8                   | 1                 |                |                   | 1               | 2                 |                 | 3                 |                 |
| 9                   |                   |                |                   | 1               | 1                 |                 |                   |                 |
| 10                  |                   |                |                   | 2               | 2                 |                 |                   |                 |
| 11                  |                   | 1              |                   | 1               | 1                 |                 |                   |                 |
| 12                  |                   |                |                   |                 | 1                 |                 |                   |                 |
| 13                  |                   |                |                   |                 | 1                 |                 |                   |                 |
| 14                  |                   |                |                   |                 |                   |                 |                   |                 |
| Dead Tested         | $\frac{1}{10}$    | $\frac{5}{10}$ | $\frac{0}{30}$    | $\frac{11}{30}$ | $\frac{0}{30}$    | $\frac{12}{20}$ | $\frac{9}{20}$    | $\frac{19}{20}$ |

Table III. The effect of temperature on the distribution of deaths with time in mice infected with graded doses of S. typhimurium, strain RIA.

## SECONDARY BACTERIAL INFECTION IN MICE

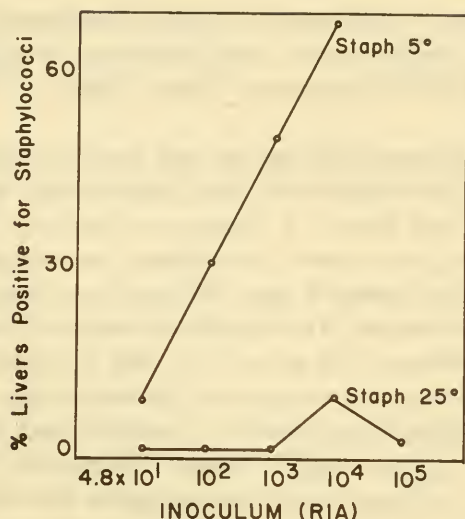


Figure 1. The per cent of liver cultures positive for staphylococci in mice at 5° C and at 25° C as related to the infecting dose of *S. typhimurium*, strain RIA. All liver cultures were made 14 days postinfection.

In order to test the possibility that a cold environment enhanced the virulence of the organism (strain RIA), isolates were recovered from the liver and spleen of infected mice sacrificed for the purpose after being held at 5° C for various periods up to 14 days. These isolates were injected into normal mice and the LD<sub>50</sub> and mean survival times were determined at both temperatures. No differences from the parent strain were noted, nor were there any detectable changes in colonial morphology or growth rate in vitro when compared to the original strain. Using these criteria, it would appear that a cold environment does not alter under in vivo conditions the virulence of the invading organism.

Table III shows the mortality rates of mice at room temperature and in the cold when given graded doses of strain RIA. It is apparent that animals maintained in the cold are not only more susceptible to infection, as judged by the increased mortality rate, but the initial deaths occur sooner than those at 25° C. When mice are injected with an LD<sub>50</sub> dose of strain RIA ( $4.8 \times 10^5$  cells per mouse at 25° C, as determined directly and not by calculation, as mentioned above),

a highly significant difference is noted in the mortality ratios between the two temperatures. This difference is significant at the 0.8 per cent level by rank test (White, 1952).

Influence of temperature on the per cent of livers positive for bacteria. At the termination of each experiment, all animals that survived the 14 day period of observation were killed by cervical dislocation. The livers were immediately excised and cultured on nutrient agar, MacConkey's agar, SS agar, and Staphylococcus 110 agar by the print method. The results are shown in Figure 1 for the experiments conducted with strain RIA. Not indicated on this figure was the observation that the per cent of mice from which salmonellae could be isolated was greater in animals kept in the cold than in those at 25° C. Moreover, as might be expected, the per cent of positive livers increased in proportion to the size of the infectious dose. Even more important was the number of mice which gave staphylococci from liver imprint cultures. This secondary invader, as can be seen from Figure 1, is to be found in animals kept at 5° C in an incidence that is proportional to the number of salmonellae (RIA) administered in the primary infection. For mice housed at 25° C, a proportionality between primary and secondary infection is not at all apparent. It would seem, therefore, that a combination of cold and salmonellosis predisposes to an invasion of staphylococci. When inocula exceed the LD<sub>50</sub> dose for mice at 5° C, few mice survive to be tested for tissue staphylococci and hence an atypical group, the highly resistant animals, survives. Thus, the relationship between primary and secondary infection was unpredictable, as indicated by the curves plotted for the 10<sup>4</sup> and 10<sup>5</sup> inocula. Despite the tendency toward selection of atypical survivors, the data obtained with livers cultured for staphylococci continued to show a substantially higher incidence of secondary invasion for mice in the cold than for animals at room temperature.

The effect of low temperature on salmonella-carrier mice. Results similar to those obtained with strain RIA were noted when the highly virulent strain SR-11 was used in experiments with mice which proved to be typhoid carriers. The results of this study, using mice which arrived from the dealer with feces that yielded positive cultures for salmonellae, are shown in Figure 2. Thus, normal mice infected with avirulent salmonellae and exposed to cold show

## SECONDARY BACTERIAL INFECTION IN MICE

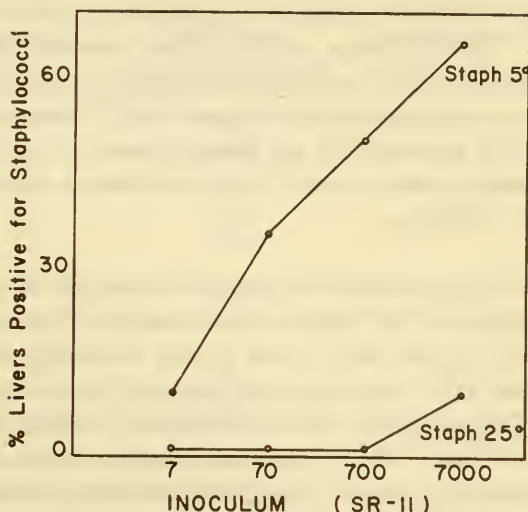


Figure 2. The per cent of liver cultures positive for staphylococci in natural salmonella carrier mice at 5° C and at 25° C as related to the infecting dose of *S. typhimurium*, strain SR-11. All liver cultures were made 14 days postinfection.

an incidence of livers positive for staphylococci in the same way as do carrier mice in the cold infected with virulent SR-11. Staphylococci were not present in livers cultured from carrier mice maintained at 25° C except those infected with the highest dose of strain SR-11, and even here only 10 per cent of the mice had a positive culture. The high degree of immunity of the carrier mice is evidenced by the fact that all animals at room temperature were able to withstand 7000 LD<sub>100</sub> of strain SR-11, while only four of 10 animals died from this dose at 5° C. The SR-11 strain is usually fatal to mice when only a single cell is injected (Schneider and Zinder, 1956). Even with enhanced resistance, some carrier animals succumbed at 5° C. The death of these mice shows that exposure to low environmental temperature increases susceptibility to infection even in highly immune mice.

It might be suggested that as increasing numbers of salmonellae are injected, progressively mounting stress is applied to a host already stressed by cold so that staphylococci already in the host or its environment now become established in its tissues. Noteworthy



also was the observation that from a group of 30 non-infected controls held at 5° C for 14 days, only 23.3 per cent had liver cultures positive for staphylococci and from none could salmonellae be obtained. A similar number of control mice at 25° C had negative liver cultures for both salmonellae and staphylococci. This further supports the contention that animals in the cold have a decreased capacity to resist infection.

Efforts have been made to determine the origin of the staphylococci which appear in the tissues of cold exposed mice. Most of the normal animals (95 per cent or 294 of 308) discharge staphylococci in the feces, but only 1 per cent approximately are coagulase-positive strains. These figures remain essentially unaltered in normal mice exposed to cold. Since coagulase-negative strains do not lend themselves readily to phage typing, a correlation between the staphylococci isolated from tissues and those found in feces of the same mouse could not, therefore, be attempted.

An indirect method was employed, however, using a modification of the technique of Schaedler and Dubos (1962) which consists of substituting 0.01 N hydrochloric acid for the drinking water in order to rid the gut of staphylococci.

Animals have been maintained on hydrochloric acid drinking water for over 40 days without any obvious untoward effects. The appearance and behavior of the animals was normal. There was normal weight gain and growth, and the pH and character of the stools was indistinguishable from that of normal controls.

Effect of acid water treatment on the per cent of mice with feces positive for staphylococci. In most experiments five to seven days of acid treatment sufficed to rid the gut completely of culturable staphylococci. In this connection, however, a seasonal effect was noted in experiments conducted during the summer months even though the animals had air-conditioned quarters. At this time of year, additional time on acid treatment was required to free the intestine of staphylococci, as shown in Table IV. Moreover, while studies conducted during the winter consistently yielded coagulase-negative staphylococci from the feces, summer studies revealed a low percentage of coagulase-positive strains as well as a high

## SECONDARY BACTERIAL INFECTION IN MICE

| Duration of<br>HCL treatment<br>(Days) | Per cent of mice<br>with fecal<br>staphylococci | Per cent<br>coagulase<br>negative | Per cent<br>coagulase<br>positive |
|--|---|-----------------------------------|-----------------------------------|
| 0                                      | 95<br>(308)                                     | 95                                | 0                                 |
| 6                                      | 67<br>(15)                                      | 67                                | 0                                 |
| 10                                     | 11<br>(27)                                      | 11                                | 0                                 |
| 12                                     | 5<br>(20)                                       | 5                                 | 0                                 |
| 28                                     | 40<br>(20)                                      | 30                                | 10                                |

Table IV. Staphylococci recovered from the feces of mice after various periods of hydrochloric acid treatment. Number of mice tested shown in parentheses.

percentage of coagulase-negative strains. When no staphylococci could be cultured from the feces after a period of acid treatment, the digestive tract was then recolonized with strain Giorgio, a coagulase-positive staphylococcus, and for about a week thereafter the stool cultures contained only coagulase-positive strains. While mice continued to shed these strains for at least several additional weeks, they also began to discharge coagulase-negative strains by the 14th day. This occurred in animals maintained at 25° C and 5° C.

Although the gut can be made free of staphylococci by treatment with hydrochloric acid, these organisms became reestablished in approximately 50 per cent of the animals within several days after tap water was substituted for the acid. In fact, in animals autopsied after the usual experimental period of 14 days, coagulase-negative staphylococci were found in nearly all stool cultures. This too was independent of the environmental temperature at which the mice were kept.

Effect of acid water treatment on the per cent of mice with tissues positive for staphylococci. Deep tissue invasion has, with only a few exceptions, consisted of coagulase-negative

## MIRAGLIA AND BERRY

| Treatment                      | Per cent of tissues positive<br>for staphylococci* |      |
|--------------------------------|--|------|
| 1 day at 5° C                  | 0  | (5)  |
| 5 days at 5° C                 | 0  | (5)  |
| 8 days at 5° C                 | 40   | (5)  |
| 14 days at 5° C                | 50   | (18) |
| 21 days at 5° C                | 40   | (5)  |
| 14 days at 25° C               | 0  | (10) |
| 10 days HCl + 5 days at 5° C   | 33   | (3)  |
| 10 days HCl + 14 days at 5° C  | 56   | (9)  |
| 10 days HCl + 14 days at 25° C | 10   | (10) |

Table V. Staphylococci recovered from tissues of mice after various treatments. All isolates were coagulase-negative. Number of mice tested shown in parentheses. \*Tissues tested: liver, kidney, spleen, lung and heart.

strains. Table V summarizes the results. The intestine when colonized with the coagulase-positive Giorgio strain could not be the origin, therefore, of the secondary invader. But even more revealing is the fact that staphylococci were still isolated from the tissues of cold exposed mice with the anticipated frequency in individuals whose feces contained no culturable staphylococci.

Reestablishment of coagulase-negative staphylococci in the gut of mice rendered free of staphylococci by acid treatment did not alter the frequency of tissue invasion by coagulase-negative organisms. This too is evidence against the possibility that the invaders are intestinal in origin.

In tissues of acid-treated cold exposed mice, a few cases were recorded in which both coagulase-positive and coagulase-negative strains were found in the same individual but only one type was present in any particular organ. In cases where coagulase-positive

## SECONDARY BACTERIAL INFECTION IN MICE

| Duration of<br>HCl treatment<br>(Days) | Per cent of mice<br>with nasal<br>staphylococci |      | Per cent<br>coagulase<br>negative | Per cent<br>coagulase<br>positive |
|--|---|------|-----------------------------------|-----------------------------------|
| 0                                      | 100   | (60) | 100                               | 0                                 |
| 10*                                    | 100   | (78) | 95                                | 5                                 |
| 12                                     | 100   | (80) | 100                               | 0                                 |
| 28*                                    | 100   | (10) | 90                                | 10                                |

Table VI. Staphylococci recovered from the noses of mice after various periods of hydrochloric acid treatment. All mice were nasal carriers of staphylococci before acid treatment. Number of mice tested shown in parentheses. \*Summer mice.

staphylococci were isolated from the tissue, the nasal flora likewise consisted of coagulase-positive strains. These data, in conjunction with those above, implicate the respiratory tract (nares) as a possible focus from which secondary invaders arise.

The data of Table V demonstrate that secondary invasion by staphylococci in normal mice exposed to cold requires approximately a week and appears to reach a maximum in 14 days. Prior treatment of the mice with hydrochloric acid drinking water did not alter significantly, moreover, either the incidence or timing of staphylococcal involvement of deep tissue.

In view of the above, and since it is well known (Taylor and Dyrenforth, 1938) that acute cold adversely affects the upper nasal passages, this area was studied to determine if it might serve as a possible portal for deep tissue invasion.

Effect of acid water treatment on the per cent of mice with noses positive for staphylococci. Table VI shows that the percentage of mice with culturable staphylococci from the nose remains essentially unaltered regardless of the experimental procedures to which the animals are subjected. For example, hydrochloric acid drinking water given for various periods up through 28 days did not lower the per cent of staphylococcal nasal carriers among normal or Giorgio fed mice. This is contrary to what was noted in the gut since it could be freed of staphylococci following acid water treatment and then re-



colonized with the Giorgio strain with great facility. Thus, nasal staphylococci which are mainly coagulase-negative are found to persist unabated in all 228 mice studied.

Nasal cultures also revealed that in the winter only coagulase-negative strains were harbored, but that in the summer a low incidence of positive strains was also evident. In a group of mice from which coagulase-positive strains were isolated from the tissues for the first time, it was found that the stools contained only coagulase-negative staphylococci, whereas also for the first time coagulase-positive organisms were isolated from the nose. Coagulase-positive strains have never been isolated from the tissues of mice which had coagulase-negative nasal flora. Thus, a correlation may exist between the nasal flora and the organisms isolated from deep tissue as secondary invaders in cold stressed mice.

Effect of acid water treatment on the per cent of salmonella carrier mice with feces positive for salmonella. It is reasonable to assume that since hydrochloric acid treatment eradicates staphylococci from the gut, the population of other members of the intestinal microflora may be likewise altered. This phase of the study has not been actively pursued, but results from a preliminary experiment using 40 mice show that the carrier rate for salmonella was reduced from 80 per cent to 5 per cent,  $P < .008$  (Wilcoxon, 1949), in 24 hours by using the acid water treatment. This indeed appears to be a dramatic reduction, but owing to the inherent shortcomings of the sampling method and the obvious danger of generalizations based on a single experiment, a more definite statement concerning the efficacy of this treatment for carrier mice must await more intensive studies.

## DISCUSSION

Host defenses are breached in animals maintained in the cold. The two levels of cellular defense, one comprised of the more peripheral wandering phagocytes and the other the deeper fixed tissues of the reticuloendothelial system (RES), are each affected by cold or

## SECONDARY BACTERIAL INFECTION IN MICE

hypothermia. Halpern et al. (1951) studied the activity of the RES, as judged by its ability to clear colloidal carbon, in hypothermic rats and found a decided reduction in function. In rats at normal temperatures, 90 per cent of the carbon was "fixed" in the RES in 35 minutes, while in the hypothermic animal 29 per cent was sequestered.

Frohlich (1938), in studies of wandering phagocytes, found an increase in number of polymorphonuclear leucocytes in hypothermic rabbits, as noted by others, but up to 65 per cent of the cells were either injured or were atypical. Similarly, Taylor and Dyrenforth (1938) reported an impairment of phagocytic activity of fixed tissue cells in human subjects immersed in water at 20.3° C. It was claimed, moreover, that low environmental temperatures predisposed to infections, especially in the upper respiratory region, but the evidence was not convincing, primarily on the basis of sample size. A decrease in blood content of complement and opsonin was found by Wildfuhr (1950) in persons exposed to cold. Thus, the humoral as well as the cellular defense is said to be altered by cold. In some host-parasite systems, therefore, low ambient temperatures are generally deleterious and seem to enhance not only an infection already underway but seem to "unmask" any secondary involvement.

Attempts to compare data obtained from various laboratories suffer, unfortunately, from the lack of adequate standardization in experimental design. That different host-parasite models are used assumes little importance in face of the realization that not all investigators report the duration of the photo period per day and the housing conditions employed. Furthermore, the term "cold", depending on the investigator, frequently spans sizeable temperature ranges. Even the conditions used in the studies just described are quite artificial and may not have a counterpart in nature. Animals were subjected to a constant and unfluctuating cold. This forces them to live at a level of high energy expenditure for long periods, a condition seldom known to occur with any certainty in nature. Moreover, the photo period was always 12 hours of light per day, and the light intensity was constant. This, too, of course, is contrary to the natural state. In spite of these apparent shortcomings, the results were constant and reproducible. Evidence for a decreased host resistance in the cold to infection with S. typhimurium is convincing and is even easier to accept in view of the increased incidence of

secondary infection with staphylococci in mice maintained in the cold.

The differences in host behavior at room temperature and at low temperature in response to salmonella infection is more apparent at infectious dosages below or at the approximate LD<sub>50</sub> level. With heavier inocula, homeostatic balance becomes erratic. This is especially true in experiments in which attempts were made to culture organs for bacteria at the 14th post-infection day. In this regard, data not included in this report indicate that while the per cent of salmonella positive livers is greater in animals held at 5° C than in those at room temperature, this difference becomes less pronounced as heavier inocula are employed. The per cent of livers positive for staphylococci, however, increase with increasing dosages of salmonellae, especially of strain RIA.

There still remain to be answered several perplexing questions. Not the least of these is the observation that while by their very nature staphylococcal infections tend to localize and form well-defined foci of infection, this has never been observed in the hundreds of animals autopsied during the course of these investigations. Gross examination of the nasal passages and sinuses failed to show a localized pathology in 14 days, the usual term of these studies. The invasion involves lung, heart, kidney, spleen and liver in an unpredictable manner and without any apparent preference for any specific tissues, this in face of the the usual course of events in which the staphylococci frequently invade the kidneys with subsequent overt signs.

Paradoxical also has been the observation that while mice fed the coagulase-positive Staphylococcus aureus, strain Giorgio, as a contaminant in their ration following acid treatment become intestinal carriers of this strain, they also become nasal carriers of the same strain, perhaps by the manner in which they eat. This state rarely lasts for more than 48 hours nor are more than 30 to 40 per cent of the mice such transient carriers. Even so, these individuals never yielded coagulase-positive isolates from deep tissue.

It would appear, then, that while a correlation seems to exist between the flora of the nose and that of deep tissue, this can not, under



## SECONDARY BACTERIAL INFECTION IN MICE

the conditions of these experiments, be altered experimentally so that a coagulase-positive strain established artificially in the host by eating infected food be subsequently made to invade deep tissue.

The manner in which infectious agents reach potential victims, enter them and establish themselves with subsequent detriment to the host has been recognized since the "Golden Era of Bacteriology". What needs further elucidation are the mechanisms responsible for the absence of overt disease symptoms in hosts parasitized by virulent pathogens which are known to persist for long periods of time.

The microorganism possessing the weapons of infectivity and pathogenicity upon entrance into a suitable host need not cause disease. This bespeaks of the complexity of the host-parasite relationship. Those studying the infectious process have long been aware that many "normal" animals harbor in their tissues a variety of parasites including viruses and bacteria. There are reports in the literature of a high incidence of the virus of polio and herpes simplex and the microbe of tuberculosis, indicating that the ability of the animal to remain free of clinical signs despite invasion exceeds its ability to prevent microbial and viral penetration. Thus, it may not be surprising that staphylococci are found in deep tissues of mice, but why its incidence is increased when the host is stressed by cold or cold and primary infection requires answer.

There is every reason to believe that there are a number of agents that may parasitize man without his knowledge and are exacerbated only during periods of stress. There are, for example, reports suggesting that herpes simplex expresses its clinical picture during physical and emotional disturbances. Likewise, clinical tuberculosis is manifested in patients stressed by poor nutrition or debilitated by another disease (primary infection). Thus, pathogens or potential pathogens can and in some instances do persist without clinical symptoms. Detection of these elusive agents depends upon adequate procedures and, therefore, come to light only after requisite advances in technology. Recovery of salmonellae from the excreta of individuals with typhoid fever, or from the blood and other tissues during certain stages of the infection, can be accomplished successfully with present bacteriological methods. However, when the host



is a carrier (asymptomatic), attempts to culture the organism become more difficult. This aspect of the problem is not unique for analogies exist in other systems. In rodents, the etiological agent of pseudotuberculosis cannot be isolated from the animal in the normal state, but can be induced to multiply rapidly in individuals under stress or those given large doses of cortisone.

It is reasonable to assume that the nature of the infected tissue itself may contribute to the difficulty attending efforts to uncover the presence of pathogens. Tissue fluids containing either antibodies or other inhibitors transferred along with the pathogen may prevent its ultimate detection not because of its absence but because its multiplication on suitable substrate is prevented. It becomes necessary under these conditions to remove the effect of the inhibitory substances either by simple dilution or by more sophisticated procedures before the pathogen can be successfully demonstrated. Since uncovering procedures are implemented only with considerable difficulty, a more indirect approach might be utilized in attempts at "unmasking"; that is, by stressing the host to a level where its influence on the parasite becomes minimized. An explosive replication of the pathogen would then permit its presence to be detected by standard procedures. A stratagem of this type successfully executed would do much to broaden our knowledge of agents whose presence otherwise escapes us.

In recent years, virologists have provided additional evidence that latent viral infections are common to man as well as animals. Data are also accumulating which suggest that latent infections of bacterial etiology may be equally common. Approximately half of the normal population harbor in their nasopharynx coagulase-positive staphylococci, and undoubtedly other agents will be detected when sought with greater effort.

That such infections exist and are capable under the proper conditions of causing overt disease more than justifies any attempts at applying in carefully controlled experiments stressors which will assist in their detection.

## SECONDARY BACTERIAL INFECTION IN MICE

### SUMMARY

1. The LD<sub>50</sub> dose for mice of S. typhimurium, strain RIA, is  $4.1 \times 10^5$  cells per mouse for animals individually housed without bedding and maintained at 25° C. It is  $3.8 \times 10^3$  cells per mouse for animals similarly housed but kept at 5° C.

2. No effect of cold could be detected in mice infected with the highly virulent SR-11 strain of S. typhimurium since all animals died following infection with only a few cells. Mice that were natural carriers of salmonellae as judged by fecal discharge were highly resistant to challenge and responded to cold in a manner similar to normal mice infected with RIA.

3. Strain RIA could be isolated from the tissues of infected animals with greater frequency and persisted longer in mice maintained at 5° C than those at 25° C.

4. Staphylococci were isolated from livers of animals that survived salmonella infection for 14 days at 5° C and the incidence of staphylococci was proportional to the number of salmonellae injected. At 25° C, only a small percentage of mice had staphylococci in tissues and these occurred independent of the infectious dose of salmonellae. These observations were made on normal mice infected with RIA and on carrier mice infected with SR-11.

5. The feeding of 0.01 N hydrochloric acid to mice in lieu of drinking water is apparently harmless to the general well-being of the animals under the conditions indicated, but rids the gut of all culturable staphylococci in five to seven days in experiments conducted in the winter. The period of hydrochloric acid treatment must be extended to achieve comparable results in summer studies. Neither ridding the gut of the normally present coagulase-negative staphylococci nor establishing a coagulase-positive strain by the feeding of contaminated food altered the incidence of tissue invasion by coagulase-negative organisms.

6. Hydrochloric acid treatment failed to alter the incidence of

nasal staphylococcal carriers. Hence, the origin of the secondary invading staphylococci appears to be the upper respiratory tract and not the gut; however, coagulase-positive strains artificially established in the nose by eating infected food could not be made to invade deep tissue.

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## DISCUSSION

NUNGESTER: Here, again, we have the problem of the hypothermic animal apparently secondarily invaded more frequently than an animal kept at the normal ambient temperature.

MIRAGLIA: Did you say hypothermic animal?

NUNGESTER: Yes.

MIRAGLIA: We try to be very careful to avoid this. The normal controls were not hypothermic, but of course when the animal is infected, the temperature does go down.

BERRY: I would just like to ask an obvious question. Should this be called a secondary infection? If infection implies any disadvantage to the host, I am not sure this is an infection in these mice. It is an interesting observation, however. Dr. Miraglia is attempting to establish coagulase-positive staphylococci in the respiratory tract to see if he can then isolate coagulase-positive staphylococci from the deep tissues. If it can be achieved

## SECONDARY BACTERIAL INFECTION IN MICE

with a highly virulent organism, then perhaps a real secondary infection can be established. This is the direction that the work is now taking, so the dream of studying secondary infections may yet come true.

ANDREWES: May I make a rather philosophical comment which applies to all the papers we have heard this morning? It seems to me that in every case, we have been presented with facts which are rather difficult to explain, and I think the reason is that, in these various instances, we don't know exactly why the animal dies when it dies, what kills it, nor do we know what saves the animal when the different mechanisms are operating. It seems to me that in every instance, if only we knew why the animal died and more details about what saved it, we would be able to isolate the various factors and pin down what it is that is affected by chilling. In Dr. Miya's paper, for instance, I was very struck with the fact that from what he reported and from what other people have reported, it didn't look as if chilling had a tremendous effect on the amount of antibody which was formed, and yet in his chilled and unadapted mice, the immunity mechanism, which on paper was perfectly adequate, failed to work. Now, why wouldn't it work; what is the difference between these two groups of mice? If we knew what sort of thing, I think we might understand how cold works. I am not suggesting that we should abandon all work on chilling until we know the answers to these fundamental questions, because I think it is possible that studying the chilling may help us to understand. What we need is for experiments going on in parallel on the mechanism which operates under normal, ordinary circumstances, combined with more experiments on the effect of chilling. I am sorry if all that appears platitudinous.

NUNGESTER: I think it is very appropriate that this statement be made, and this point of view be brought out. Just ask a simple question: why do you have a fever when you have an infection?

CAMPBELL: Well, along the same lines, there are mechanisms of defense other than antibody, of course. So I was wondering what happens to the phagocytic cells and the lymphocytes? There

## MIRAGLIA AND BERRY

must have been some studies along this line.

MIRAGLIA: There have been some studies along these lines, and it appears that there is an increase in the number of phagocytes when an animal is subjected to cold, but their capacity to phagocytize and digest is greatly impaired. No, we have tried to mimic the effect of cold by using known RES suppressants, such as Proferrin, and we have been somewhat successful in this, but we need to do a great deal more work.

BERRY: Just by chance, I have a slide. This shows carbon clearance following intravenous injection into mice. Almost complete clearance is accomplished in about thirty minutes. Animals kept at 5° C for two hours and eighteen hours were injected with carbon and compared with mice housed at 25° C. There is not a dramatic difference, but it is statistically significant. We have no other data at the present time which evaluates the effect of cold on the activity of the reticuloendothelial system. I was talking with Derrick Rolly of the University of Adelaide in Montreal, and he suggested that we use labeled bacterial cells rather than carbon; then, he said, we would probably get a completely different type of result. We shall certainly try to do this very promptly now.

NUNGESTER: These results were in what animal, and for how long had it been chilled?

BERRY: The straight line applies to two different groups; mice chilled for two hours, and mice chilled for eighteen hours. The two-hour time period and the eighteen-hour time period gave similar results. We used the two-hour time period because by then the body temperature has dropped.

MIYA: I'd like to answer Sir Christopher. I don't want to sound like I keep harping on this psychological effect, but if you will recall from the slide with respect to the Klebsiella pneumoniae, the isolated mice, singly caged and immunized, were not protected under acute cold stress. These mice are subject to a stress of cold, a stress of isolation, and a stress of challenge, which makes a total of three stresses; whereas, their counter-

## SECONDARY BACTERIAL INFECTION IN MICE

parts housed in groups have only the stress of cold, plus the stress of challenge. If you talk in terms of how many stresses are actually applied to the experimental animal, it may be a quantitative thing that you could put your finger on. Let's say it is the function of the total stress as applied to the organism, and I think this can be tested by taking a singly-caged animal and placing it at room temperature in a cage which has an electrical charge to it, then you have the three stresses. You can induce as many more stresses as you see fit.

VIERECK: Dr. Miya, do I understand you to say that isolation is a stress? Usually, in theories of population dynamics, densities, as they get greater, are considered to be more stressful. I know you are dealing with animals in the cold, but it sounded like you considered isolation, per se, without cold, to be a stress to an animal. What is your evidence?

MIYA: Well, I don't have any for an animal, but I think in terms of human beings.

VIERECK: High population density has been studied as a stress in humans.

MIYA: I think I could survive longer in Alaska with one other person to talk to than just by myself.

MITCHELL: Mice are happy only when they're alone.

MIRAGLIA: I don't wish to minimize the psychological effect, but we have conducted experiments with both group-housed and isolated animals at room temperature, and the LD<sub>50</sub> is not altered, so using this criteria alone, which, to be sure, is not enough by itself, there is no psychological effect of isolation.

REINHARD: It seems to me that the investigators here are facing the extreme difficulty of separating the effect of one type of flora from another. And you have to go to extreme kinds of manipulations, like feeding hydrochloric acid to rid a beast of one or other organisms. I wonder, in this day and age of germ-free animals, whether the latter would not be more suitable



subjects for the study of the effect of individual species of bacteria in individual animals.

NUNGESTER: That would be very interesting except for the price.

BERRY: The germ-free animal is a highly artificial animal, let me say.

REINHARD: That is true. And so is the animal that is rid of any one part of the flora by the rather rigorous means that have been described.

PREVITE: Getting back to Sir Christopher's comments and Dr. Campbell's comments, in a very light vein I would like to mention that work has been done by some Hungarian workers. They have reported on studies of the complement titers, and phagocytic capacity of Guinea pigs after acclimatization to cold. The animals were housed outdoors during winter and warmer seasons of the year. I was very excited about these papers some time ago. However, their third paper strongly implied that those animals housed outdoors in the cold passed on greater immunity to disease because of the inheritance of acquired characteristics.<sup>1</sup>

BERRY: Coming back to Sir Christopher's remark about the immunized mice showing an effect of cold, whereas the non-immunized mice do not. Cold has a subtle influence on host-parasite interaction, and if one is dealing with a highly virulent organism that is certain to kill the animal, then cold will not modify this relationship. If one has a relationship that is more nearly in balance, one that can go either way, then cold can tip the balance and produce an effect either way. The cause of death in an infectious disease is another point, and something that we all need to know more about. There are a few people foolish enough to work on this problem, and I hope more will tackle it, because ultimately, infectious diseases must be understood at a metabolic level. As difficult as it is, I would like

<sup>1</sup> Szemere, Gy., A. Bodi, and L. Csik, 1960, *Academiae Scientiarum Hungaricae Tomus X*.

## SECONDARY BACTERIAL INFECTION IN MICE

to make a plea that everyone keep the problem in mind, and whenever there is a chance, throw light on it.

